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DISSERTATION

„Long term cellular immune response to prophylactic human papillomavirus (HPV) vaccines"

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1 INTRODUCTION

1.1 HPV infection

1.1.1 Papillomaviruses

Papillomaviruses are a family of small DNA viruses that are able to infect only epidermal or mucosal epithelia.[1] They are non-enveloped particles consisting of a 55 nm long icosahedral capsid with 72 pentameric capsomers in a skewed (T=7) arrangement. The capsomers are made out of two structural proteins, a major capsid protein also known as late protein 1 (L1) and a minor capsid protein or late protein 2 (L2), required for virion assembly.[1-3] The capsid carries a double-stranded circular DNA genome that contains approximately 8000 base pairs and can be divided into three distinct regions. The two coding regions contain early genes, E1, E2, E4, E5, E6 and E7, which encode non-structural proteins or the late genes which encode the two capsid proteins. The third, non-coding region, known as the long control region (LCR), contains most of the regulatory elements involved in viral DNA replication and transcription.[4,5]

Papillomaviruses are strictly species-specific, and of interest to us are only the human papillomaviruses (HPV).[1] To date, more than 130 HPV genotypes have been cloned from different clinical lesions. At least 40 of them can infect the anogenital epithelia. According to their oncogenicity, these can be further divided into high-risk types, which are associated with high-grade dysplasias as well as cervical and other anogenital cancers, and low-risk types, responsible for low-grade dysplasias and condylomata acuminata. Worldwide epidemiological studies have classified thirteen different oncogenic HPV types into the high-risk group, namely HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 66. HPV 26, 53, 68, 73 and 82 should be considered probably carcinogenic. A further eleven HPV types, namely HPV 6, 11, 40, 42, 43, 44, 54, 61, 70, 72 and 81, among others, belong to the low-risk group.[2,6-8]

HPV-associated diseases play an important role in many clinical disciplines, including gynaecology, dermatology, urology, proctology and otorhinolaryngology. The emphasis of this thesis will be on female genital HPV infections.

1.1.2 Epidemiology

Infections with human papillomaviruses are among the most common sexually transmitted infections. Though common, HPV infections are often transient and most of the infected

individuals are able to eliminate the evidence of the virus without ever developing clinically recognized manifestations.[9-12]

Most sexually active individuals are likely to be exposed to HPV during their lifetimes and acquire HPV infection. According to a meta-analysis, which included 78 studies, worldwide HPV prevalence in women with normal cytology at any given point in time is approximately 10,4%, with a higher overall prevalence in less developed countries.[11] The great variation of HPV prevalence in various studies can be partially explained by differences in the sensitivity of the assays used for the detection of HPV DNA as well as by differences in the age range and socio-cultural background of the study population.[9]

HPV infection is most common in women younger than 25 years old, decreasing with age thereafter despite continuously high levels of sexual activity. A possible explanation for this finding lies in the development of the memory immune response which provides protection against recurrent HPV infections. A second peak in HPV prevalence is seen in the peri- or postmenopausal years. Plausible explanations for this phenomenon include reactivation of latent infections due to gradual impairment of the immune response, possibly caused by various hormonal changes at this age, and acquisition of new infections due to changes in sexual behaviour of individuals in middle age. However, HPV prevalence across age is subject to great variations, which appear largely to reflect the differences in sexual behaviour across geographical regions.[9-11]

Epidemiological data indicate that more individuals are infected by high-risk HPV types than by low-risk types. The five most commonly acquired HPV types worldwide are HPV 16, 18, 31, 58 and 52. As many as 32% of all infected women with normal cytological findings are positive for HPV 16 or 18, or both. The relative contribution of these two HPV types is substantially higher in severe clinical lesions, possibly due to their great tendency to persist and progress to cervical lesions (see Chapter 1.2.1). It can be assumed that HPV 45 shares this tendency as it belongs to the five most common HPV types detected in invasive cervical cancer but is much underrepresented in cytologically normal women.[11,13,14]

1.1.3 Ethiopathogenesis

1.1.3.1 Transmission

Anogenital HPV infections are easily transmissible. The predominant route of transmission is via sexual contact with an infected partner, including also non-penetrative sexual activity.[5,12] A

Danish study including 100 virgins showed that 35% of women who initiated sexual activity acquired an HPV infection within four years, whereas no evidence of HPV infection could be detected among women who remained virgins.[15] However, there is evidence indicating that HPV transmission occasionally occurs through skin contact with contaminated objects, as well as perinatally from infected mother to child. Although possible, dreaded perinatal transmission is generally a rare event.[5,16]

The major risk factor for the transmission of HPV infection is the increasing number of lifetime sexual partners.[5,15,17] The age at first sexual intercourse is inversely correlated to the risk of HPV infection, which may reflect either the association between the young age at sexual debut and more frequent change of sexual partners, or the greater susceptibility of the anogenital epithelia during adolescence.[18] Further risk factors include smoking,[19,20] parity,[21] increased use of oral contraceptives rather than condoms,[22,23] other sexually transmitted infections,[24-26] immunosuppressive conditions, such as HIV infection,[27] nutrition,[28] and polymorphisms in the human leukocyte antigen (HLA) system,[29] though results regarding their importance for the acquisition of the HPV infection are inconsistent.[5,10]

1.1.3.2 Viral replication cycle

HPV infects primitive basal keratinocytes, probably stem cells, at the sites of micro-abrasions of the epidermal or mucosal epithelia. The following viral replication cycle is absolutely dependent on the complete differentiation of the squamous epithelia. The viral gene expression in primarily infected cells is largely suppressed. Although limited, the expression of oncogenes, specifically E6 and E7, results in enhanced proliferation of these cells and their ascension in the suprabasal layers. Parallel to the differentiation of the infected keratinocyte in stratum spinosum and granulosum of the squamous epithelia, expression of both early and late viral genes is massively up-regulated. As a result, the circular viral genome is replicated and structural proteins L1 and L2 form. Finally, complete infectious viral particles are assembled in stratum corneum and released as the keratinocytes die off.[30] The newly formed viral particles can now potentially re-infect the individual's epithelium, or can be transmitted to naive individuals.

In high-risk lesions, a significant role for the malignant transformation of the infected keratinocytes is assigned to the two major oncoproteins E6 and E7. The degradation of the tumour suppressor pRb and cyclin-dependent kinase inhibitors p21 and p27 following their interaction with E7 results in resistance to apoptosis and an increase in chromosomal instability.[4,31] E6 on the other hand supports the immortalization of the infected cell by degrading

the pro-apoptotic proteins p53 and BAK.[4,32] Indefinite proliferation of these immortalized cells is provided by E6-induced activation of the telomerase. In addition, interaction of E7 with pRb and its related proteins releases several transcription factors from the E2F family, which in turn stimulate the expression of proteins essential for the cell cycle progression, such as cyclins A and E.[4] The resulting accumulation of DNA damage facilitates the production of potentially transformed progeny.

1.1.3.3 Viral persistence

HPV has several features that enable it to avoid host defence systems. During the infection with these strictly epitheliotropic viruses there is no viremia, and only minimal amounts of replicating virus are exposed to immunocompetent tissue. The HPV replication cycle previously described induces neither cytolysis nor necrosis, and therefore no inflammation. There is consequently little or no release of the pro-inflammatory cytokines necessary for the activation and migration of antigen presenting cells (APCs).[4,30] In addition, E6 limits the presentation of viral antigens to the professional APCs of the squamous epithelia, known as Langerhans cells, by reducing the number of adhesion molecules (E-cadherin) on the surface of keratinocytes.[33] Interaction of the two major oncoproteins, E6 and E7, with interferon regulatory factors inhibits the synthesis of two potent antiviral immunomodulators, interferon (IFN) α and β, further favouring the viral persistence. In addition, these oncogenes inhibit the expression of a toll-like receptor in high-risk lesions, which is responsible for recognition of pathogens' double-stranded DNA sequences.[4,30]

Despite the efforts of the virus to evade host defences, 80 to 90% of all genital HPV infections are cleared in approximately 6 to 18 months.[10,34-36] This is achieved due to a successful cell-mediated immune response directed against early HPV proteins, particularly against E2 and E6, followed by formation of specific antibodies against the major capsid protein, L1. However, following a natural infection only a weak induction of the humoral immunity can be detected, and many women do not seroconvert.[30] About 10 to 20% of individuals with a genital HPV infection do not become DNA negative. The resulting persistent infection is a prerequisite for HPV-associated diseases.[37]

1.2 HPV-associated diseases

1.2.1 Invasive cervical cancer and its precursor lesions

Based on a number of clinical observations on malignant transition of condylomata acuminata into squamous-cell carcinoma, Harald zur Hausen postulated the role of HPV in the

carcinogenesis of cervical cancer.[38] In 2008 he received the Nobel prize for proving the association between HPV and anogenital diseases.[39]

Cervical cancer represents the second most common malignancy in women around the world, with over 80% of cases occurring in countries without organized cytology screening.[40] According to the cancer registry it represents the 13th most common malignancy in women in Germany. In 2006 no fewer than 5470 women developed cervical cancer and 1492 died from the consequences of this disease. Most commonly affected are women between the ages of 40 and 59.[41]

Persistent infection with high-risk HPV is considered to be a prerequisite for the development of cervical cancer and its precursor lesions. HPV DNA can be found in almost all cervical cancer biopsies.[14,39,42] Approximately two-thirds of all cervical cancers worldwide can be attributed to infection with HPV 16 and 18 (61% and 10%, respectively). HPV 45 and 31 are the next two most common HPV types found in cervical cancer biopsies, together causing approximately 10% of cervical cancers worldwide.[14]

Approximately 85% of all cervical cancers are squamous-cell carcinoma and up to 15% are adenocarcinoma. The incidence of adenocarcinoma has increased in most developing countries, particularly among younger women.[43] HPV 18 and 45 DNA is more frequently found in this disease than in squamous-cell carcinoma (32% and 12%, respectively, in adenocarcinoma compared with 8% and 5%, respectively, in squamous-cell carcinoma).[14]

High-risk HPV types preferentially infect cervical transformation zone epithelia, which are subject to continuous changes during a woman's life.[4] As previously described (see Chapter 1.1.3.2), expression of high-risk E6 and E7 oncoproteins is essential for immortalization and enhanced proliferation of the infected cells, resulting in instability of the cellular genome. In addition these two oncoproteins induce centrosome abnormalities, generating mitotic defects and possibly even aneuploidy of the cellular genome.[44] The accumulation of various mutations in the cellular genome can lead to malignant transformation of the infected epithelia. With increasing severity of cervical lesion, HPV DNA integration into the cellular genome becomes more frequent. The expression of E6 and E7 oncoproteins is up-regulated in cells harbouring the integrated viral genome, facilitating further their malignant transformation.[45]

Fortunately, the process of malignant transformation is slow and requires several years from first infection to the development of invasive cervical cancer, allowing for the implementation of

secondary prevention programmes. Within the framework of the cancer prevention programme in Germany, every woman over the age of 20 is supposed to undergo annual cytology screening with the Papanicolaou (Pap) test, which is financed by the health insurance companies. [2] Detected cytological lesions need either close monitoring by cytology, further diagnostic by colposcopy and histological examination of cervical biopsies, or ablative therapy.[10]

According to the Bethesda cytological classification, cervical lesions detected by the Pap test can be divided into atypical squamous cells of undetermined significance (ASCUS), or low-grade and high-grade squamous intraepithelial lesions (LSIL, HSIL).[46] The histological equivalent of LSIL is the cervical intraepithelial neoplasia grade 1 (CIN 1, see Fig. 1). CIN 1 is a mild dysplasia, in which the atypical cells occupy the lower third of the epithelium. It appears to represent a transient manifestation of a productive HPV infection with a high rate of spontaneous regression, making its role as a cancer precursor uncertain. HSIL include CIN 2 and CIN 3, or moderate and severe dysplasia including carcinoma in situ. In high-grade lesions atypical cells extend beyond the lower third of the epithelium and frequently show abnormal mitoses and aneuploidy. HSIL are considered to be the true precursors of cervical cancer.[5,9,46] A prospective study of 31 women who had carcinoma in situ showed that 72% of them developed cervical cancer within 12 years of follow-up.[47]

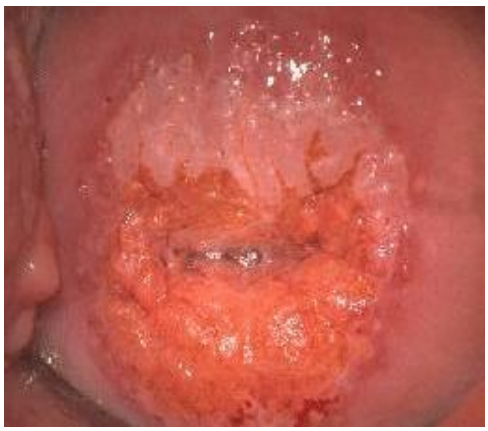

Fig. 1: Cervical intraepithelial neoplasia, CIN [3]

1.2.2 Other female anogenital cancers

It is well established that persistent infection with HPV is the cause of almost all cases of cervical and also a substantial proportion of other female anogenital cancers. Overall the high-risk HPV type most commonly found in anogenital cancers and their respective precursor lesions is HPV 16.[5] Up to 50% of vulval cancers are HPV positive and these seem to have a better prognosis in comparison with HPV negative tumours of the same site. HPV DNA can be detected in up to 90% of vaginal and anal cancers.[39,48] Cancer of the perianal skin on the other hand resembles vulval cancer in its lower prevalence of HPV DNA positivity.[5] Vulval and vaginal intraepithelial neoplasia (VIN, VaIN) grade 2 and 3 are considered to be the true precursors of the respective invasive cancers. These lesions are much less common than CIN 2

and 3, but the treatment is difficult, can result in disfigurement and requires long-term follow-up due to the frequent recurrence of the disease.[49]

1.2.3 Condylomata acuminata

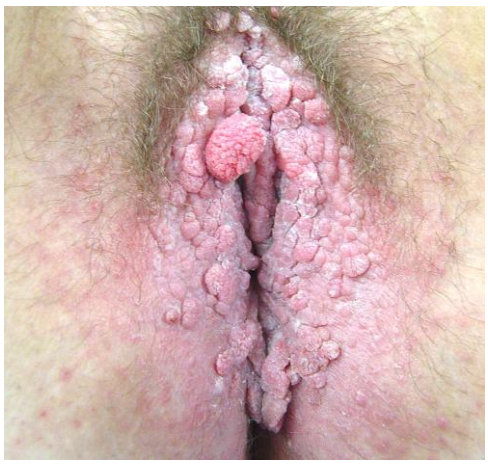

Fig. 2: Condylomata acuminata [3]

Condylomata acuminata (see Fig. 2), also known as genital warts, represent benign lesions of the anogenital epithelia which affect about 1% of all sexually active individuals between the ages of 15 and 45.[10] Most commonly affected are women between the ages of 20 and 24. Genital warts are caused by persistent infection with low-risk HPV types, HPV 6 and 11 being responsible for approximately 90% of all cases of genital warts.[50,51] In most women infection with low-risk HPV types remains subclinical. A study of 512 university students in the USA showed that only one out of four women infected with HPV 6 or 11 actually developed genital warts.[52]

According to the Bethesda cytological classification, genital warts belong to LSIL.[46] In contrast to lesions caused by persistent infection with high-risk HPV types, there is nearly no risk of malignant transformation of genital warts. Low-risk oncoproteins effectively enhance epithelial proliferation, but show much lower transforming activity and are incapable of inducing genomic instability.[44]

Approximately 30% of lesions regress spontaneously, and others can be removed by various modes of treatment.[2] Due to the common persistence of HPV infection even after the removal of lesions, recurrence of the disease occurs in most of the treated individuals.[2] Genital warts produce no symptoms at all, or cause itching, burning or tenderness of the affected area and dyspareunia. Although not life threatening, these lesions may present a substantial emotional and social burden for infected women.

1.3 Prophylactic HPV vaccines

1.3.1 Gardasil® and Cervarix™

When expressed from a heterologous promoter in an eukaryotic cell, the major capsid protein L1 of the papillomaviruses has an intrinsic capacity to self-assemble into virus-like particles

(VLPs).[53] Recombinant VLPs are morphologically indistinguishable from authentic viral capsids, but they contain no viral genome and are therefore not infectious. Evidence collected using various animal models shows that, when administered intramuscularly or subcutaneously, VLPs are able to induce high titers of type-specific neutralizing antibodies. Passive immunization with purified serum IgG from VLP immunized animals protected naive animals from subsequent homologous viral challenge, indicating that neutralizing antibodies provide sufficient protection against HPV infection.[54-56] Based on this knowledge prophylactic HPV L1 VLP vaccines, Gardasil® and Cervarix™, have been developed (see Fig. 3).

	GARDASIL®	CERVARIX™
Structure	HPV L1 virus-like particles (VLP)	
	6 / 11 / 16 / 18 20 µg / 40 µg / 40 µg / 20 µg	**16 / 18** 20 µg / 20 µg
Adjuvans	**AAHS** (225 µg of amorphous aluminium hydroxyphosphate sulfate)	**AS04** (500 µg of aluminium hydroxide plus 50µg of 3-dacylated monophosphoryl lipid A)
Substrate	Yeast (Saccharomyces cerevisiae)	Baculovirus expression system (Spodoptera frugiperda Sf-9 and Trichopulsia ni Hi-5 cell substrate)
Injection schedule	3 intramuscular injections in the deltoid area at	
	day 0, month **2** and 6	day 0, month **1** and 6

Fig. 3: Comparison of the two prophylactic HPV vaccines [57,58]

The quadrivalent vaccine Gardasil® (Sanofi Pasteur-Merck Sharp Dohme) is composed of L1 VLPs of HPV 6, 11, 16 and 18, synthesized via recombinant technology in Saccharomyces cerevisiae. To enhance the immunogenicity, purified VLPs were adsorbed onto a proprietary amorphous aluminium hydroxyphosphate sulphate adjuvant (AAHS).[57] The bivalent vaccine Cervarix™ (GlaxoSmithKline) contains L1 VLPs of HPV 16 and 18 produced on Spodoptera frugiperda Sf-9 and Trichopulsia ni Hi-5 cell substrate.[58] Its adjuvant system, AS04, combines aluminium hydroxide and 3-deacylated monophosphoryl lipid A (MPL), an agonist of the toll-like receptor 4 (TLR4), resulting in rapid and spatially localized activation of the innate immune response, which in turn contributes to the development of much stronger adaptive immunity.[58,59] Both vaccines are administered intramuscularly and the complete recommended vaccination cycle is comprised of three doses.

Due to convincing evidence from clinical trials, in Germany Gardasil® and Cervarix™ have been licensed for use on boys and girls older than 9 years of age since October 2006 and October 2007, respectively. STIKO (Ständige Impfkommission am Robert-Koch-Institut) officially recommends vaccination of all girls aged 12 to 17, preferably prior to sexual debut.

1.3.2 Immunogenicity

Virtually all women who completed the vaccination cycle with either one of the two prophylactic vaccines and were naive to respective vaccine-type HPV at the study enrolment have seroconverted. The peak geometric mean titers (GMT) for vaccine-induced antibodies were observed upon the completion of the three-dose regimen. These were 10- to 100-fold higher than antibody titers detected after natural infection. Following this peak response, vaccine-induced antibody titers declined over time, reaching a plateau between months 18 and 24 and remaining stable thereafter. [57,58,60-63] Antibody titers against HPV 16 and 18 measured 5 to 6 years after the vaccination with Cervarix™ were at least 13- and 12-fold higher, respectively, than those detected after clearance of a naturally acquired infection.[61] It is important to mention that 5 years after vaccination with Gardasil® a proportion of women were found to be seronegative for one or more vaccine-type HPV, most commonly HPV 18. However, no breakthrough cases of confirmed infection with, or disease caused by, the vaccine-type HPV were reported through 4.5 years of follow-up.[64]

Until recently, direct comparison of serological data from clinical trials of the two vaccines was not possible due to differences in the detection assays used. A recent study including 1106 women aged 18 to 45 showed that GMTs of antibodies against HPV 16 and 18 measured at month 7 by pseudovirion-based neutralization assay (PBNA) were 2- to 9-fold higher after vaccination with Cervarix™ compared with Gardasil®. In addition, higher antibody concentrations in the cervicovaginal mucus and stronger memory-specific B cell responses were detected in Cervarix™ recipients.[65]

1.3.3 Efficacy of the prophylactic vaccines

Cervarix™ and Gardasil® efficacy is documented for 9.4 and 5 years, respectively.[62,66] Tables 1 and 2 summarize the main results on the efficacy of these two prophylactic vaccines from multiple randomized, placebo-controlled, double-blind phases II and III clinical trials involving healthy women aged 15 to 26.

Table 1: Prophylactic efficacy of GARDASIL® [49,57,62,67-70]

CLINICAL TRIAL	Mean follow-up (months)	Study population (verum/ placebo)	Endpoint	GARDASIL® efficacy				
				ATP[1]	ITT[2]		TVC[3]	
				Vaccine type HPV	Vaccine type HPV	Any HPV type	Vaccine type HPV	Any HPV type
Villa, 2005 [57]	36	276/ 275	HPV persistance (≥4 months)	89	88	NR	NR	NR
			CIN 1-3	NR	100	NR	NR	NR
			Anogenital disease	100	100	NR	NR	NR
Villa, 2006 [62]	60	276/ 275 In extended follow-up 114/ 127	HPV persistance (≥4 months)	96	94	NR	NR	NR
			CIN 1-3	100	100	NR	NR	NR
			CA	100	100	NR	NR	NR
			Anogenital disease	100	100	NR	NR	NR
Garland, 2007 (FUTURE I) [67]	36	2723/ 2732	CIN 1-3, AIS	100	98	NR	55	20
			CA, VIN 1-3, VaIN 1-3	100	95	NR	73	34
Villa, 2007 (FUTURE II) [68]	36	5305/ 5260	CIN 2/3, AIS	98	95	NR	44	17
Ault, 2007† [69]	36	9087/ 10292	CIN 2/3, AIS	99	98	NR	44	18
Joura, 2007‡ [49]	36	7811/ 7785	VIN 2/3, VaIN 2/3	100	97	NR	71	49
Muños, 2010 (FUTURE I and II) [70]	42	8689/ 8702	CIN 1	NR	98˚ *	30˚ *	69	20
			CIN 2	NR	100˚ *	43˚ *	55	19
			CIN 3	NR	100˚ *	43˚ *	45	16
			AIS	NR	100˚ *	100˚ *	60	63
			CA	NR	96˚ *	83˚ *	80	62
			VIN 2/3, VaIN 2/3	NR	95˚ *	77˚ *	79	51

CIN / VIN / VaIN: cervical / vulval / vaginal intraepithelial neoplasia; **CC / VC / VaC:** cervical / vulval / vaginal cancer; **AIS**: adenocarcinoma in-situ; **CA:** condylomata acuminata; **Anogenital disease:** CA, CIN 1-3, AIS, CC, VIN 1-3, VC, VaIN 1-3 and VaC; **NR:** not reported;

[1]) **„According To Protocol":** women who received all three doses of the vaccine or placebo, had no major protocol violations, were seronegative and HPV DNA negative to vaccine-type HPV at baseline and remained HPV DNA negative up to 1 month after the third dose;

[2]) **„Intention To Treat":** women who received at least one dose of the vaccine or placebo, were seronegative and HPV DNA negative to vaccine-type HPV at baseline;

[3]) **„Total Vaccinated Cohort":** all women enrolled in the study who received at least one dose of the vaccine or placebo, regardless of their HPV status or presence of HPV-associated anogenital disease at the study enrolment;

˚ in addition women were HPV DNA negative to all high-risk HPV at baseline;

***** only including women with normal cytology at baseline;

† combined analysis of three Gardasil® studies and one study with HPV 16 L1 VLP vaccine;

‡ combined analysis of three Gardasil® studies.

Table 2: Prophylactic efficacy of CERVARIX™ [58,60,61,71-74]

CLINICAL TRIAL	Mean follow-up (months)	Study population (verum/ placebo)	Endpoint	CERVARIX™ efficacy				
				ATP[1]	ITT[2]		TVC[3]	
				Vaccine type HPV	Vaccine type HPV	Any HPV type	Vaccine type HPV	Any HPV type
Harper, 2004 [60]	18-27	560/ 553	HPV persi stance (≥6 months)	100 ˚ *	NR	NR	88	NR
			≥ ASCUS	NR	NR	NR	93	NR
Harper, 2006 [58]	48	560/ 553 In extended follow-up 393/ 383	HPV persi stance (6 months)	96 ˚ *	94 ˚ *	NR	NR	NR
			HPV persistance (12 months)	100 ˚ *	94 ˚ *	NR	NR	NR
			≥ ASCUS	NR	96 ˚ *	48 ˚ *	NR	40
			CIN 2/3, CC	NR	100 ˚ *	67 ˚ *	NR	52
Romanowski, 2009 [61]	76	560/ 553 In extended follow-up 393/ 383	HPV persi stance (6 months)	100 ˚ *	NR	NR	NR	NR
			HPV persistance (12 months)	100 ˚ *	NR	NR	NR	NR
			≥ ASCUS	NR	97 ˚ *	35 ˚ *	NR	NR
			CIN 2/3, CC	NR	100 ˚ *	72 ˚ *	NR	NR
Caravahlo, 2010 [71]	88	560/ 553 In extended follow-up 222/ 211	HPV persi stance (6 months)	100 ˚ *	NR	NR	NR	NR
			HPV persistance (12 months)	100 ˚ *	NR	NR	NR	NR
			≥ ASCUS	NR	97 ˚ *	41 ˚ *	NR	NR
			CIN 2/3, CC	NR	100 ˚ *	41 ˚ *	NR	NR
Paavonen, 2007 [72]	15	9319/ 9325	HPV persistance (6 months)	NR	80	NR	NR	NR
			HPV persistance (12 months)	NR	76	NR	NR	NR
			CIN 2/3, CC	NR	90	NR	NR	NR
Paavonen, 2009 [73]	35	9319/ 9325	CIN 2/3, CC	98	98 ˚ *	70 ˚ *	53	30
			CIN 3, CC	100	100 ˚ *	87 ˚ *	34	33
Roteli-Martins, 2011 [74]	100	436 (verum and placebo)	HPV persistance (12 months)	100	NR	NR	NR	NR
			CIN 2/3, CC	100	NR	NR	NR	NR

CIN / VIN / VaIN: cervical / vulval / vaginal intraepithelial neoplasia; **CC:** cervical cancer; **ASCUS:** atypical squamous cells of undetermined origin; **≥ASCUS:** ASCUS, low-grade and high-grade intraepithelial lesions; **NR:** not reported;

[1]) **„According To Protocol":** women who received all three doses of the vaccine or placebo, had no major protocol violations, were cytologically negative, seronegative and HPV DNA negative to vaccine-type HPV at baseline and remained HPV DNA negative up to 1 month after the third dose;

[2]) **„Intention To Treat":** women who received at least one dose of the vaccine or placebo, were HPV DNA negative to vaccine-type HPV at baseline;

[3]) **„Total Vaccinated Cohort":** all women enrolled in the study who received at least one dose of the vaccine or placebo, regardless of their HPV status or presence of HPV-associated anogenital disease at the study enrolment;

˚ in addition women were HPV DNA negative to all high-risk HPV at baseline;

***** only including women with normal cytology at baseline.

In the according-to-protocol (ATP) cohort, both vaccines have shown up to 100% efficacy regarding any given endpoint of the respective trial, indicating very strong protection of HPV naive women against persistent infection with, and anogenital disease caused by, the vaccine-type HPV. Comparable results were found in the intention-to-treat (ITT) cohort, which included all women who received at least one dose of vaccine or placebo and were seronegative and HPV DNA negative to vaccine-type HPV at baseline. The efficacy was lower if the population analysed consisted of all women enrolled in the trials, including those not vaccinated according to the protocol and those with baseline evidence of past or current HPV infection (total-vaccinated-cohort, TVC).[49,57,58,60-62,67-74] It is interesting to note that Cervarix™ provides 70% protection against CIN 2+ regardless of the responsible HPV type in HPV naive women of the ITT cohort, dropping to still impressive 30% in the TVC cohort.[73] The efficacy of Gardasil® with respect to the same lesions in this latter population is approximately 18%.[69]

Approximately 6% of all gynaecological cancers are of vulval or vaginal origin. Detection of these cancers and their precursor lesions relies solely on visual and bimanual examination, since no screening programmes for these malignancies exist. The combined analysis of three clinical trials with Gardasil® showed 71% efficacy against high-grade VIN or VaIN caused by the vaccine-type HPV in the TVC cohort.[49] The vaccination with Cervarix™ should theoretically provide similar protection.[75]

The efficacy of Gardasil® with respect to prevention of condylomata acuminata caused by HPV 6 and 11 was shown to be 100% in the ATP and 71% in the TVC cohort of a large phase III clinical trial.[67] Since L1 VLPs of the two low-risk HPV types are only included in Gardasil®, Cervarix™ is not expected to show prophylactic efficacy against infection with, and disease caused by, these two types.

1.3.4 Cross-Protection

Both prophylactic vaccines have shown evidence of cross-protection against non-vaccine HPV types. Using 6 month persistent infection as the most relevant endpoint, Cervarix™ efficacy was shown to be 79% against HPV 31, 76% against HPV 45 and 46% against HPV 33 in women vaccinated according to protocol, who were DNA negative for the corresponding HPV type at baseline. Gardasil® has shown a 46% efficacy only against HPV 31. The efficacy against high-grade CIN lesions caused by HPV 31, 33, 45, 52 and 58 in the same cohort was 53% and 29% for Cervarix™ and Gardasil®, respectively.[73,76]

1.4 Vaccine-induced protective immunity

1.4.1 Innate and adaptive immunity

The human immune system is characterized by innate and adaptive immunity. The innate immune system (phagocytes, natural killer cells, complement, cytokines) detects the pathogen in a non-specific manner and provides immediate defence against the infection. Its role in the activation of the adaptive immune system is crucial.

The adaptive immune system consists of a humoral and a cellular arm. Unlike the innate immune system, the adaptive immune system is able to generate antigen-specific responses and to provide specific immunological memory. Following a natural infection or vaccination, protective immune responses can be established, which are able to generate a more rapid recall of the immune effectors upon re-exposure to the same antigen, thereby protecting the host from re-infection or preventing persistence of an infection.

The humoral arm of the adaptive immune system is comprised of B lymphocytes (B cells), which are able to transform into plasma cells and secrete antigen-specific neutralizing and cytotoxic antibodies.

Although humoral immunity mediated by the neutralizing antibody responses to L1 proteins is considered to be the hallmark of protection induced by the prophylactic HPV vaccines, the cellular immune responses must not be forgotten. T lymphocytes (T cells) act as mediators of cellular immunity. Based on the surface marker they are bearing (CD = cluster of differentiation), the T cells can be divided into CD4+ T helper cells and CD8+ cytotoxic T cells (CTL).[30,77,78]

1.4.2 VLP-induced humoral immune response

Following intramuscular injection, vaccine VLPs are rapidly phagocytosed by circulating antigen presenting cells (APC), processed and, after migration to the local lymph nodes, presented on the major histocompatibility complex class II molecules (MHC II) for recognition by naive CD4+ T cells. The complex interactions with the VLP-loaded APC result in activation of the naive CD4+ T cells and their differentiation into CD4+ effector T cells, namely T helper 1 (Th1) or T helper 2 (Th2) cells. These subsets of the effector T cells are defined on the basis of different cytokines that they secrete (see Chapter 1.4.3).[30,78,79]

Unlike T cells, B cells recognize the vaccine VLPs in their native conformation. The antigen binding virtually always only primes the naive B cells. In order to further differentiate, the primed B cells require co-stimulatory signals, which are provided by the activated Th2 and, to a minor extent, Th1 cells (see Fig. 4). With the help of MHC II, the primed B cells present processed VLP to the activated T helper cells, which use their T cell receptor (TCR) and CD4 to bind on the MHC II-VLP-derived peptide-complex. The bond between the B and the T cells is further strengthened by interaction between CD40 and CD154, also known as the CD40 ligand. In addition to these receptor-ligand interactions, the activated T helper cells produce cytokines (among others IL-2 and IL-4), to further support the differentiation programme of the B cells, which results in the generation of antibody secreting plasma cells. A proportion of the activated B cells will become memory B cells, which are characterized by consistent, long-term, low-level antibody production.[77,79,80]

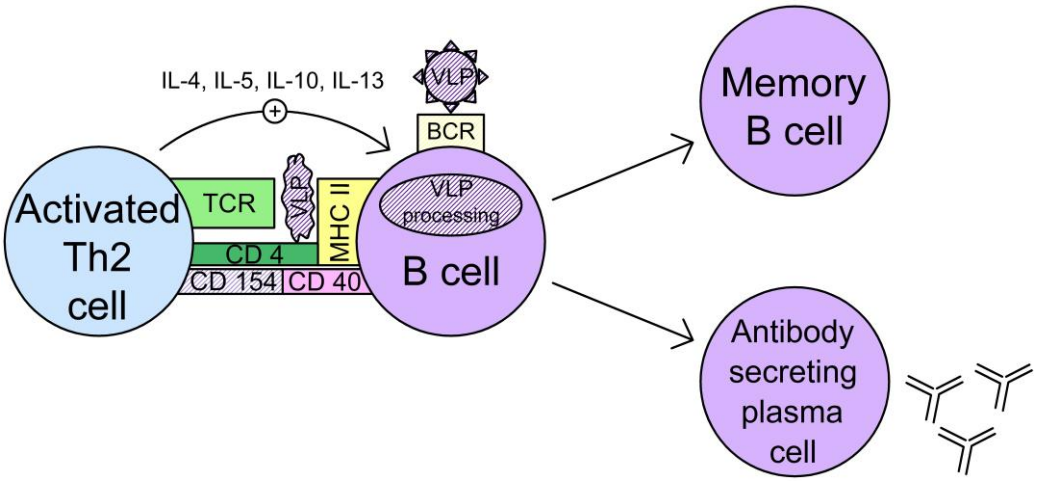

Fig. 4: Differentiation of the primed B cells

The figure shows complex interaction between the activated Th2 cell and the primed B cell, which results in further differentiation of the latter.

IL: interleukin; **Th2:** T helper 2 cell; **TCR:** T cell receptor; **CD:** cluster of differentiation; **VLP:** virus like particles; **MHC:** major histocompatibility complex; **BCR:** B cell receptor.

Secreted antibodies to L1 proteins circulate in the blood, reach the vaginal mucus and basal epithelia via secretion or transudation, respectively, thus providing protection against subsequent viral challenge.[81]

L1 proteins are structurally complex antigens containing many different epitopes or antigenic determinants, some of which are immunodominant. Following stimulation with such a complex antigen, several plasma cell clones are generated, resulting in overall polyclonal or heterogeneous antibody response.[30] Due to the high degree of L1 homology (HPV 18 and 45 L1 are approximately 88% identical, HPV 16 and 31 L1 approximately 83%) a large number of

epitopes are likely to be shared between different HPV types.[82-84] It has been suggested that the immunodominant epitopes induce high titers of type-specific neutralizing antibodies. The epitopes shared between closely-related HPV types are likely to be less immunogenic, but are able to induce cross-neutralizing antibodies which confer protection against infection with these HPV types (see Chapter 1.3.4).[30]

1.4.3 Cellular immune response

The activation of the naive CD4+ T cell is a result of a complex interaction between the T cell receptor (TCR) together with the CD4 molecule on one side and the VLP-derived peptide presented by the APCs via MHC II on the other. Additional co-stimulation of the T cell surface molecule CD28 via B7 molecules on the APCs is necessary.[80] Upon activation, the T helper cell expresses CD154 on its surface. This glycoprotein belongs to the tumor necrosis factor (TNF)-related ligand family and is considered to be the activation marker of the antigen-specific T helper cells.[85] The activated T helper cells have the ability to drive their own division by autocrine secretion of interleukin-2 (IL-2) and up-regulation of the IL-2 receptor expression.[80]

Under the influence of IL-12 produced by the APCs, the activated T helper cells differentiate into Th1 cells. These secrete IFNγ, IL-2 and TNFα and are able to induce a strong cell-mediated CTL response. Interestingly, IFNγ is considered to be an effector cytokine, being able to maintain the Th1 cell response and to exert direct cytotoxic effects. Th2 cells secrete IL-4, IL-5, IL-10 and IL-13 and, as previously mentioned, play a significant role in the induction and maintenance of the humoral responses. Further, the activated T helper cells can become T memory cells, which mainly produce IL-2, and upon re-exposure to the same antigen differentiate more rapidly and efficaciously into the T effector cells.[78] In summary, the T helper cells are responsible for orchestrating and directing the immune response.

CTLs, also known as killer cells, recognize with the help of their TCR and CD8 molecule the antigens bound to MHC I, which are present on all nucleated cells. In order to become activated, the CTLs require co-stimulation, which is provided either by the interaction of the CD28 on their surface with the B7 on the APCs, or by the cytokines secreted by the Th1 cells.[80] Once activated, CTLs express CD137, also known as 4-1BB, on their surface. CD137 is a member of the TNF-related ligand family and its cross-linking enhances the proliferation of the CTLs.[86] The activated CTLs are able to lyse infected cells using two major mechanisms, the perforin granule exocytosis pathway and the FasL/Fas mechanism of apoptosis. In addition, they secrete multiple cytokines, among others IFNγ and TNFα, thereby enhancing their anti-microbial effector

function. In analogy to the T helper cells, a proportion of the activated CTLs can become memory CTLs (see Fig. 5).[78]

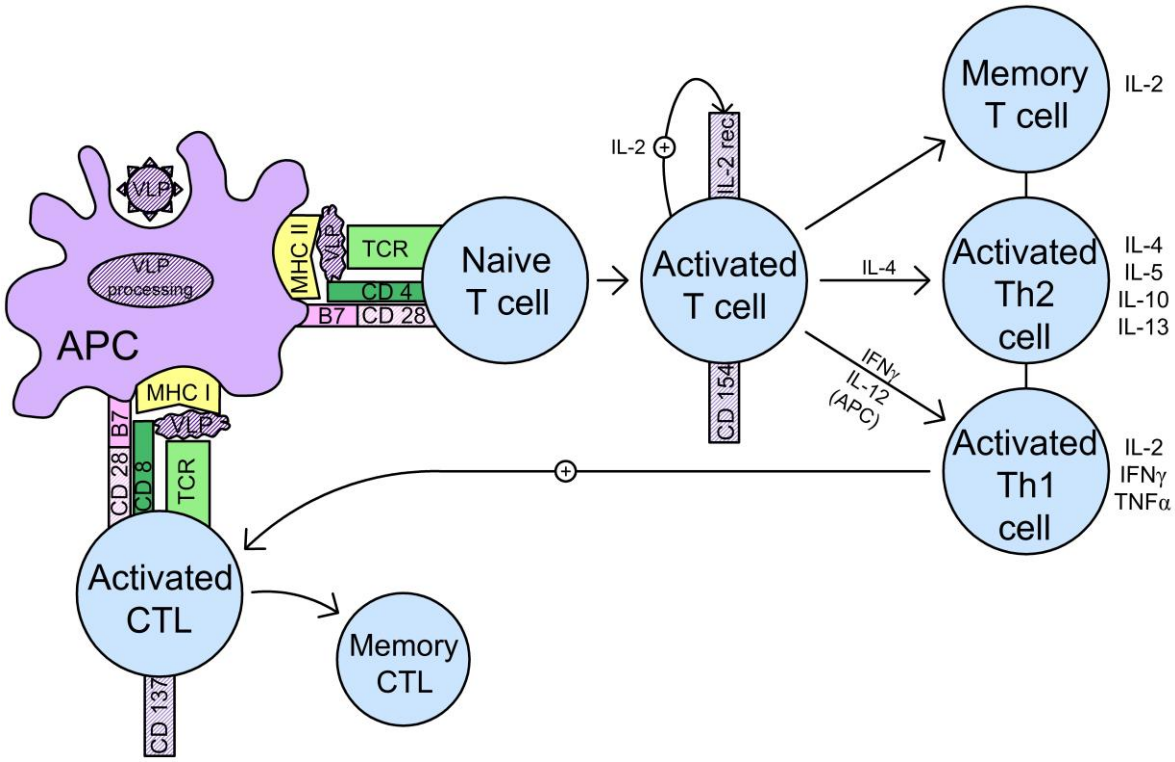

Fig. 5: Differentiation of the T lymphocytes

The figure shows complex interaction between the APC and the T lymphocytes, which results in further differentiation of the latter.

VLP: virus like peptides; **APC:** antigen presenting cell; **MHC:** major histocompatibility complex; **TCR:** T cell receptor; **CD:** cluster of differentiation; **IL:** interleukin; **IL-2 rec.:** interleukin-2 receptor; **IFNγ:** interferon-γ; **Th1/2:** T helper 1/2 cell; **TNFα:** tumor necrosis factor α.

1.5 Aims of the study

The two prophylactic HPV vaccines, Gardasil® (HPV 6, 11, 16 and 18) and Cervarix™ (HPV 16 and 18), have been on the market in Europe since 2006 and 2007, respectively. Long-term follow-up studies of both vaccines have shown high immunogenicity on the B cell level as well as efficacy of up to 100% against persistent infection with, and anogenital disease caused by, the vaccine-type HPV. In addition, both vaccines have shown evidence of cross-protection against homologous HPV types. To date, only a few studies have analysed the cellular immune response induced by the HPV vaccines.

The overall aim of this immunologic study is to analyse and compare for the first time the long-term humoral and cellular immune responses induced by the immunization with Gardasil® or Cervarix™ on average 4.5 years prior to the study enrolment.

Aim 1:

- Determine the frequency of the antigen-specific T helper cells against HPV 6, 11, 16 and 18 as well as against HPV 31 and 45 in whole blood from the study participants with the help of an *ex vivo* assay and:
- Analyse the T cell frequencies within each group separately, in order to quantify the differences in the cellular immune response to various HPV types;
- Compare the antigen-specific T cell frequencies between the Gardasil® and the Cervarix™ groups;
- Compare the data from our study with the data reported by SK Pacher 2011 on the frequencies of the antigen-specific T helper cells detected prior to the vaccination and 6 months after the completion of the full vaccination cycle with Gardasil® or Cervarix™.

Aim 2:

- Expand and detect at higher frequencies the antigen-specific T helper cells and the cytotoxic T cells against HPV 6, 11, 16, 18, 31 and 45 in cultures containing peripheral blood mononuclear cells from the study participants with the help of an *in vitro* assay and:
- Analyse the T cell frequencies within each group separately;
- Compare the T cell frequencies between the Gardasil® and the Cervarix™ groups;
- Compare the T cell frequencies detected using the *ex vivo* and the *in vitro* assay and thereby determine the expansion indices;
- Compare the expansion indices between the Gardasil® and the Cervarix™ groups.

Aim 3:

- Determine the antigen-specific antibody titers in the serum samples from the study participants with the help of the VLP-capture ELISA assay and:
- Analyse the antibody titers within each group separately;
- Compare the antibody titers between the Gardasil® and the Cervarix™ groups;
- Analyse the correlation between the humoral immune response and the cellular immune response detected by the *ex vivo* assay.

2 METHODS

2.1 Study design

To assess the immune response to the two vaccines, we conducted a non-randomised, cross-sectional study including women, immunised with either Gardasil® or Cervarix™ on average 4.5 years (SD = 1.1) prior to the study enrolment.

Inclusion criteria were limited to female gender, adult age and completion of the full vaccination cycle consisting of three doses of either Gardasil® or Cervarix™ at least 3.5 years prior to the study enrolment. Women who were underage, pregnant or breastfeeding, or who had a known history of non-HPV-related infectious diseases or psychiatric conditions were excluded from the study.

The study, including all recruitment materials and informed consent protocols, was approved on the 2nd of October 2009 by the ethics committee of the Charité – Universitätsmedizin Berlin (EA04/036/07). The ethics committee defined the study as a purely observational study, thereby excluding interventions of any kind (e.g. HPV tests or Pap smears).

The majority of women included in the study were immunised within the frameworks of phase II/III clinical trials of the two vaccines. The recruitment of 35 subjects therefore took place in cooperation with the FUTURE (Females United to Unilaterally Reduce Endo/Ectocervical disease) study site at the Virchow Klinikum, Charité – Universitätsmedizin Berlin, as well as with the GSK (GlaxoSmithKline) study sites at the Benjamin Franklin Klinikum, Charité – Universitätsmedizin Berlin, and at the Stiftung Juliusspital, Würzburg. In cooperation with Dr. Perlitz, a gynecologist in a private practice from Haldensleben, further 5 women were recruited.

Prior to blood sampling, all participants provided written informed consent. The five women from Haldensleben and the 16 women from Würzburg were recruited and educated about the study course, risks involved, and possibility of withdrawal from the study by Dr. Perlitz and Prof. Dr. Schwarz, respectively, who also conducted the blood sampling for these participants.

Altogether blood samples from 40 subjects were collected for immunologic assays at least 3.5 years after the immunisation with either Gardasil® (16 subjects) or Cervarix™ (24 subjects). Allocation of study participants and assays performed are shown in Figure 6.

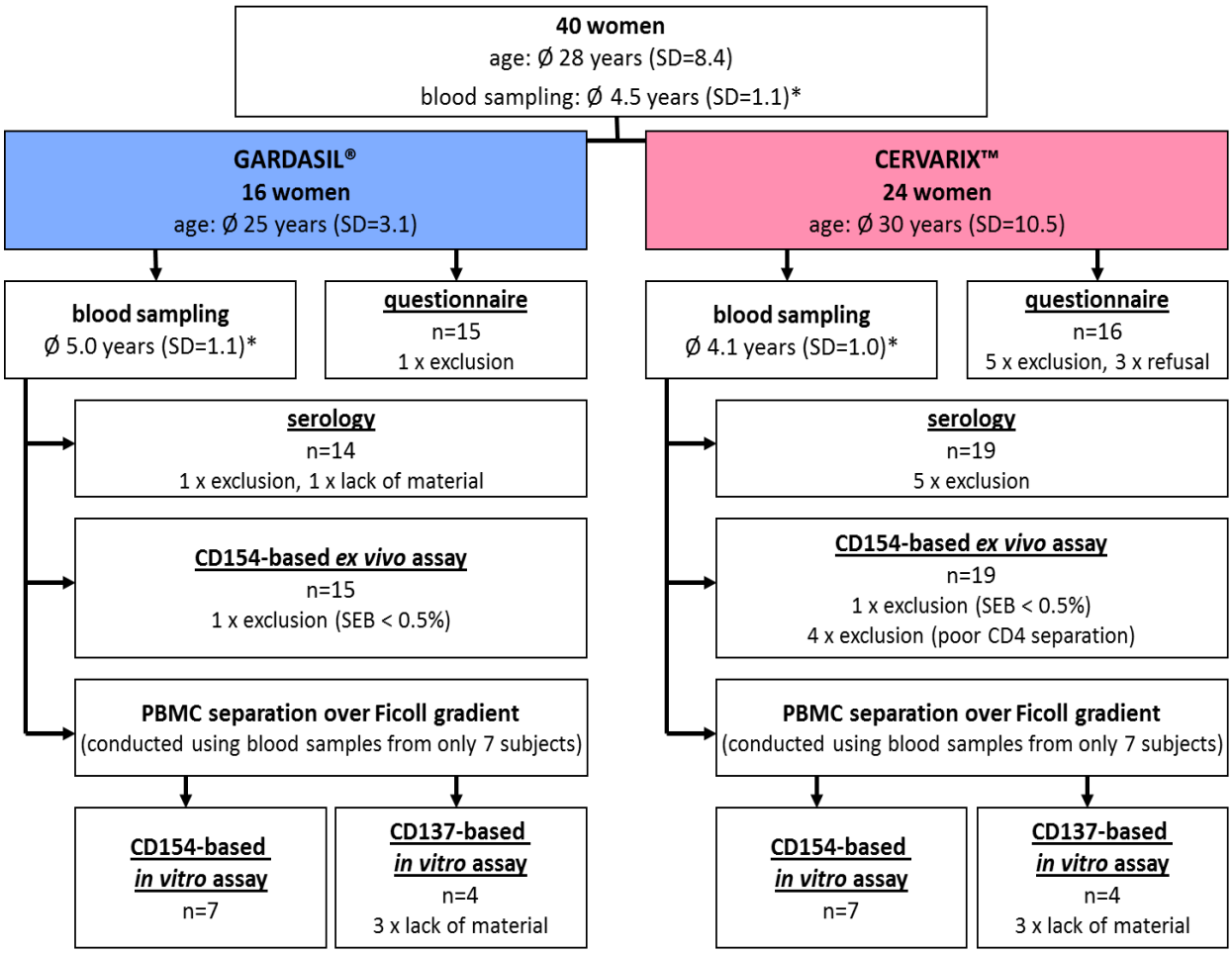

Fig. 6: Study design

The figure shows the allocation of the study participants and assays performed, as well as the reasons for the exclusion from the study population.

SD: standard deviation; **SEB:** staphylococcal enterotoxin B; **CD:** cluster of differentiation; **n:** number of subjects analysed; **PBMC:** peripheral blood mononuclear cells;

* time between completion of the full vaccination cycle consisting of three doses of either Gardasil® or Cervarix™ and blood sampling.

Using whole blood from each subject, we conducted the CD154-based *ex vivo* assay to detect the vaccine-specific memory CD4 T cells (see Chapter 2.3). For various reasons, explained in Chapter 3.2.1, 6 subjects were excluded from the study population. Peripheral blood mononuclear cells (PBMCs) isolated by separation over Ficoll gradient from whole blood taken from 14 subjects, including 7 from the Gardasil® and 7 from the Cervarix™ group, were used in the CD154-based *in vitro* assay. In addition PBMCs from 8 subjects, including 4 from the Gardasil® and 4 from the Cervarix™ group, were used to conduct the CD137-based *in vitro* assay. The goal of these assays was to expand and thereby detect at higher frequencies vaccine-specific memory CD4 and CD8 T cells (see Chapter 2.2.2 and 2.4). In order to compare the humoral and the cellular immune response, serological measurements of the HPV-specific

antibody titres were carried out (see Chapter 2.6). We were unable to perform antibody titer measurements for one subject as the serum sample was missing. In addition, a pre-formulated questionnaire was used to define more closely the study participants (see Chapter 2.7).

2.2 Materials

2.2.1 Sample collection

50 ml of whole blood was collected from each subject by peripheral venous puncture using the BD Vacutainer® system. The Vacutainer® tubes used were primed with lithium-heparin, resulting in direct anticoagulation of collected blood samples. Once collected, the blood samples were stored at room temperature and processed within the next 24 hours. The blood samples collected in Würzburg and Haldensleben were shipped to Berlin by overnight-express post and processed the next morning in our laboratory. A part of each blood sample was used for the CD154-based *ex vivo* assay and the rest was processed to PBMCs and cryopreserved (see Chapter 2.2.2). In addition, plasma samples were separated and cryopreserved for future antibody titer determinations.

2.2.2 Peripheral Blood Mononuclear Cells (PBMC)

PBMCs were isolated from heparinised whole blood by Ficoll-Hypaque (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation. The cells were then cryopreserved at temperatures between -80 and -160°C in a freezing medium, consisting of RPMI-1640+GlutaMAX (GIBCO, Karlsruhe) supplemented with 20% fetal calf serum (FCS, Biochrom, Berlin) and 10% dimethyl sulfoxide (DMSO, Sigma, Taufkirchen). Later thawed PBMCs were re-suspended in an RPMI medium consisting of RPMI-1640+GlutaMAX supplemented with 10% FCS and 1% penicillin-streptomycin (Biochrom), and used for the CD154/CD137-based *in vitro* assays.

2.2.3 Stimulation antigens

In order to activate the vaccine-specific memory T cell responses in the blood from both Gardasil® and Cervarix™ vaccinees, T cells must be stimulated *ex vivo* with the exact same antigen that was responsible for their vaccine-induced differentiation from naive into the specific T cells. The HPV capsid protein L1 represents the immunogenic component of both vaccines. Therefore, synthetically produced peptide pools comprising the complete antigen sequence of the L1 wild type proteins from HPV 6, 11, 16 or 18 were used for *ex vivo* and *in vitro* stimulation. In

addition, L1 peptide pools from HPV 31 and 45 were used for *ex vivo* and *in vitro* stimulation in order to analyse the potential activation of the cross-reactive immune response to these two high-risk HPV types. Each L1 peptide pool consisted of 28 to 31 different peptides (peptides&elephants, Nuthetal), which were made out of 30 amino acids (30mer peptides) and shared a 12mer sequence overlap between each other. In this manner all possible linear epitopes were presented to the T cells for recognition, and variations in the L1 antigen sequence as well as potential contaminations caused by the yeast or baculovirus proteins contained in the vaccines were avoided.

Following a natural infection in contrast to the vaccination, specific T cells against the HPV oncogenic proteins E6 and E7, among others, are induced. To account for prior infections with the vaccine-type HPV and the T cell responses induced by them, we stimulated the subjects' blood *ex vivo* and *in vitro* with synthetically produced peptide pools, containing the complete antigen sequence of the E6 and E7 proteins from HPV 6 (low-risk E6/E7 peptide pool) or HPV 16 and 18 (high-risk E6/E7 peptide pool). These peptide pools were also made up of multiple 30mer peptides.

30mer peptides were dissolved in DMSO at a concentration of 50 mg/ml. In order to make 500 µl of peptide pool, 10 µl of each 30mer peptide of the respective HPV type was used and the rest of the volume was made up with DMSO. These were later diluted with phosphate buffered saline (PBS) without magnesium or calcium (Biochrom) resulting in a final concentration of 1 mg/ml, and used for stimulation.

Enterotoxin B produced by the bacterium Staphylococcus aureus, (SEB; Sigma), the general viability indicator of the cell culture, was used as a positive control at a concentration of 1 mg/ml in PBS.[85]

2.2.4 Brefeldin A

Upon activation, cytotoxic T cells and T helper cells express CD137 and CD154 respectively, and in addition produce multiple cytokines. To secure the future detection of these surface markers and cytokines, it is necessary to block their degradation and secretion in the meantime.[85] Brefeldin A is a lactone antibiotic produced by a fungus called Eupenicillium brefeldianum. It inhibits, specifically and reversibly, the protein transport from the endoplasmic reticulum to the Golgi apparatus, resulting in intracellular protein accumulation.

Following the instructions of the manufacturer, Brefeldin A (Sigma) was dissolved in DMSO and diluted with RPMI medium to a final concentration of 1 mg/ml which was later used for secretion inhibition.

2.3 Ex vivo assay

The CD154-based *ex vivo* assay is based on the publication by Frentsch et al.[85] and was adapted to the HPV-specific T cell quantification assay by AK Ramseger, who established the protocol described below.[87]

Regardless of the number of samples, the antigen stimulation was conducted in the same manner: Under sterile working conditions 1 ml of whole blood was added to each cell culture tube (Greiner bio-one, Frickenhausen). These were then supplemented with agonistic anti-human CD28 (BD, Klon CD28.2) at a concentration of 1 µg/ml in order to provide for co-stimulation of the T helper cells by binding on the T cellular CD28 (see Chapter 1.4.3).

The first sample was used as a negative control and was supplemented with no further reagents. At a concentration of 10 µg/ml, L1 peptide pools of HPV 6, 11, 16 or 18 were added to the respective cell culture tubes. For five women immunised with Gardasil® and six women immunised with Cervarix™ additional two samples were stimulated with L1 peptide pools of HPV 31 or 45 at a concentration of 10 µg/ml. Samples stimulated with E6/E7 peptide pools of HPV 6, or 16 and 18, again at a concentration of 10 µg/ml, served as "natural infection" controls. The positive control was stimulated last, after other tubes had been closed, in order to avoid contamination of the other samples. It was supplemented with 5 or 10 µg/ml of SEB. Differences in SEB concentrations used for the stimulations were due to the lack of material. Therefore quantitative comparisons of these samples could not be preformed. SEB stimulations were only used to show the viability of the vaccinee's cells.

In order to enable the exchange of gas, all culture tubes were only loosely closed. The culture tubes were then placed in an ankle rack and incubated at 37°C in an atmosphere containing 5% CO_2 at a humidity of 92%. After 90 to 120 minutes, Brefeldin A was added at a concentration of 10 µg/ml. The incubation was then continued for another 10 to 22 hours.

To end the stimulation, 2 mMol of ethylenediaminetetraacetic acid (EDTA, pH 7) was added to each sample and left for 10 minutes to act. This led to detachment of the macrophages from the wall of the culture tubes. Next, whole blood erythrocytes were lysed for 10 minutes using 9 ml of

diluted FACS lysing solution (BD, diluted 1:10 with aqua dest). Lysing buffer supernatants were separated following a 10 minute centrifugation (1400 rpm) and the remaining cell pellets were washed with 2 ml of PBS each. Washed cell pellets were fixed with 500 µl of Formalin (2% in PBS) per sample. After 17 minutes the cells were washed twice with 2 ml FACS buffer, that consisted of PBS supplemented with 0.1% (w/v) of bovine serum albumin fraction V, BSA (Biomol, Hamburg) and 1% (w/v) of sodium acid. The fixed washed cell pellets were then either re-suspended in FACS buffer and stored for up to three weeks at 4 °C, or directly stained.

In order to allow intracellular staining, the fixed cells were permeabilized with 500 µl of diluted FACS permeabilizing solution 2 (BD, diluted 1:10 with aqua dest). After 10 minutes the cells were washed with 2 ml of FACS buffer and the cell pellets were then re-suspended in 400 µl of FACS buffer each. The cell suspensions were then transferred into the respective FACS tubes (BD Falcon) and centrifuged, leaving the cell pellets ready for intracellular staining with fluorochrome conjugated antibodies to CD4, CD154, IFNγ and IL-2 or IL-4.

The staining mix consisted of beriglobin (1 mg/ml in PBS; ZLB, Behring) diluted 1:50 with FACS buffer, anti human CD4 (clone SK3, BD) conjugated with peridinin chlorophyll protein (PerCP) and diluted 1:5 with FACS buffer, anti human CD154 (clone 5C8, Miltinyi Biotec) conjugated with allophycocyanin (APC) and diluted 1:10 with FACS buffer, anti human IFNγ (clone B27, BD) conjugated with fluorescein isothiocyanate (FITC) and diluted 1:100 with FACS buffer, and anti human IL-2 (clone MQ1-17H12, BD) or anti human IL-4 (clone 8D4-8, BD) both conjugated with phycoerythrin (PE) and diluted 1:100 with FACS buffer. The staining mix which contained antibodies to CD4, CD154, IFNγ and IL-2 was used to stain all samples. In addition, the negative control samples and the samples stimulated with L1 peptide pools of HPV 6, 11, 16 or 18 from 11 women immunised with Gardasil® and 13 women immunised with Cervarix™ were stained with the staining mix which contained antibodies to CD4, CD154, IFNγ and IL-4.

50 µl of the respective staining mix was added to the cell pellet in each FACS tube. These suspensions were incubated at room temperature in the dark for 30 minutes. Dispensable antibodies were washed out using 1 ml of FACS buffer. The stained cell pellets were then either re-suspended in 100 µl of Formalin (1% in PBS) and stored for up to one week at 4°C, or resuspended in 250 µl of FACS buffer and directly analysed by flow cytometry (see Chapter 2.5).

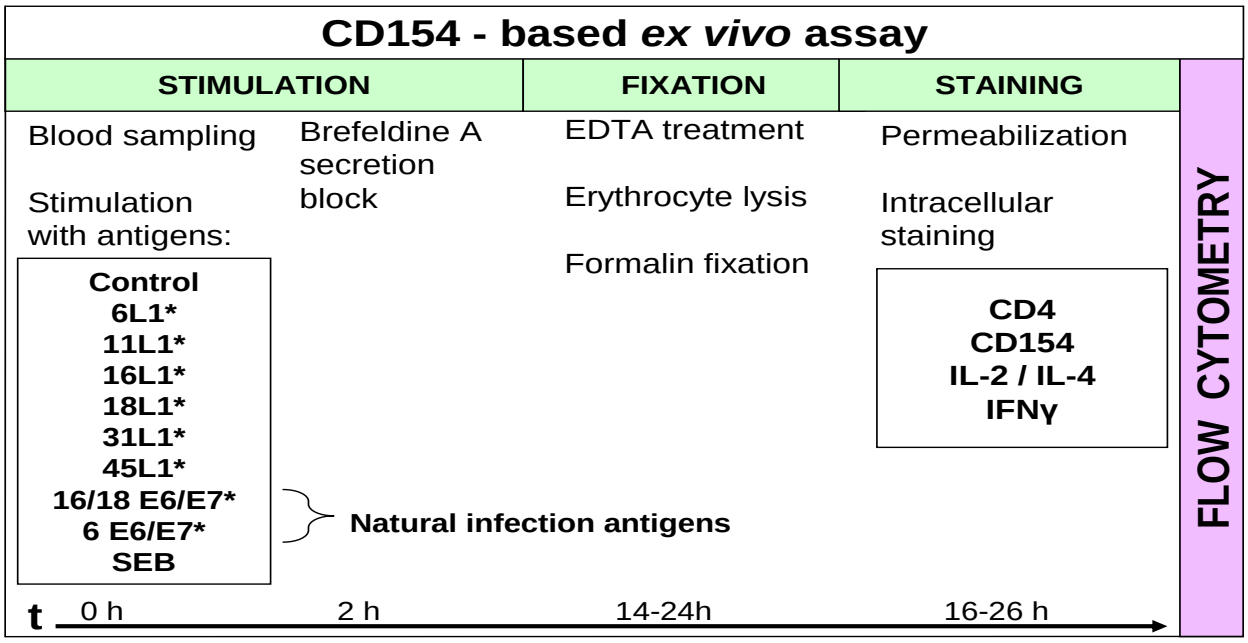

Fig. 7: Schematic representation of the CD154-based *ex vivo* assay
(*Adapted acc. to Frentsch et al., Nat. Med. 2005* [85])

CD: cluster of differentiation; **EDTA:** ethylenediaminetetraacetic acid; **L1:** late protein 1; **E6/E7:** early protein 6/7; **SEB:** staphylococcal enterotoxin B; **IL-2/4:** interleukin-2/4; **IFNγ:** interferon gamma;

* human papillomavirus peptide pools.

2.4 In vitro assay

It has been shown that antigen stimulated PBMCs have the ability to proliferate in 7- to 21- day T cell cultures, thereby allowing more sensitive detection of a higher number of the antigen-specific memory T cells.[88,89] Based on this knowledge we established a CD154/CD137-based *in vitro* assay described below, with the goal to expand and thereby detect at higher frequencies vaccine-specific memory CD4 and CD8 T cells. As previously mentioned, this assay was conducted using the PBMCs from 7 subjects from the Gardasil® and 7 subjects from the Cervarix™ group.

On day 0, the cryo-preserved PBMCs were washed twice in RPMI medium and the remaining cell pellet was re-suspended in the same medium. The volume was adjusted to the number of wells into which PBMCs were seeded. As only 50-80 % of the PBMCs survive the process of cryo-preservation, more cells were thawed than were actually needed for the *in vitro* assay. Re-suspended PBMCs were seeded into multiple wells (1 ml of cell suspension per well) on a 24-well culture plate (BD). The goal was to have approximately 1.5 x 10^6 cells per well. Each well was supplemented with IL-2 (20 IU/ml) and IL-7 (10 IU/ml).

The first well acted as the medium control and was supplemented with no further reagents. The next four wells were stimulated with L1 peptide pools of HPV 6, 11, 16 or 18 (10 µg/ml). For 3 of the 7 subjects from both groups, two additional wells were supplemented with L1 peptide pools of HPV 31 or 45 (10 µg/ml), thereby enabling the detection of the cross-reactive memory T cells against these two high-risk HPV types in the *in vitro* settings. For the other 4 of the 7 subjects from the two groups, two additional wells were supplemented with E6/E7 peptide pools of HPV 6 or 16/18 (10 µg/ml) in order to further analyse the memory immune response induced by a possible natural infection and detect the ability of the memory T cells induced by this infection to expand. Stimulated PBMC cultures were subsequently incubated for 9 to 10 days at 37°C in an atmosphere containing 5% CO_2 at a humidity of 92%.

On day 3, 500 µl of supernatant from each well was removed and replaced with the same volume of fresh RPMI medium. In cases in which the cell culture showed strong proliferation, supernatant was left in the well, 1 ml of RPMI medium was added and the culture was subsequently split into two wells. All the wells were finally supplemented with IL-2 (20 IU/ml) and IL-7 (10 IU/ml).

On day 6, 50% of the culture medium was once more replaced with fresh medium. This time no cytokines were added.

On day 9 or 10, the PBMCs were re-suspended thoroughly and transferred into respective culture tubes. The volume in each tube was adjusted to 3 ml by adding RPMI medium and the suspension was supplemented with 1 µl/ml of anti human CD28. Next, the cell suspensions were each divided into three tubes: the first was used for re-stimulation with the respective peptide pool (10 µg/ml); the second acted as a negative control and was supplemented with no further reagents; and the third acted as a positive control and was stimulated with SEB (5 or 10 µg/ml) once the other tubes had been closed. The cell suspension from the medium control well needed to be divided into two tubes only, as no re-stimulation with HPV peptide pools was necessary.

All the culture tubes were subsequently placed in an ankle rack and were incubated for 90 to 120 minutes, prior to the addition of Brefeldin A (10 µg/ml). After a further 12 to 20 hours, EDTA was added to each tube and the cells were fixed and stained in analogy to the *ex vivo* settings (see Chapter 2.3). In contrast to whole blood used in the *ex vivo* settings, the PBMC cultures contained no erythrocytes. Therefore, following the EDTA treatment, the erythrocyte lysing step was skipped and the cells were directly washed in PBS. In order to detect CD4 and CD8 positive T cells, the fixed and washed cells were re-suspended in 800 µl of FACS buffer

and then transferred into two FACS tubes, one for the intracellular staining with the staining mix containing anti human CD4, CD154, IL-2 and IFNγ described above (see Chapter 2.3) and the other for intracellular staining with the staining mix consisting of beriglobin diluted 1:50 with FACS buffer, anti human CD8 (clone SK1, BD) conjugated with PerCP and diluted 1:5 with FACS buffer, anti human CD137 (clone 4B4-1, Miltenyi Biotec) conjugated with PE and diluted 1:10 with FACS buffer, anti human IL-2 (clone MQ1-17H12, BD), conjugated with APC and anti human IFNγ (clone B27, BD) conjugated with FITC, both diluted 1:100 with FACS buffer. This additional staining was performed for only 4 of the 7 subjects from the two groups, as the results showed that in our *in vitro* settings no sufficient expansion of the CD8 positive T cells could be achieved (see Chapter 3.3.1). The samples re-stimulated with SEB were used to test the viability of the cell cultures and were only stained with anti human CD4, CD154, IL-2 and IFNγ.

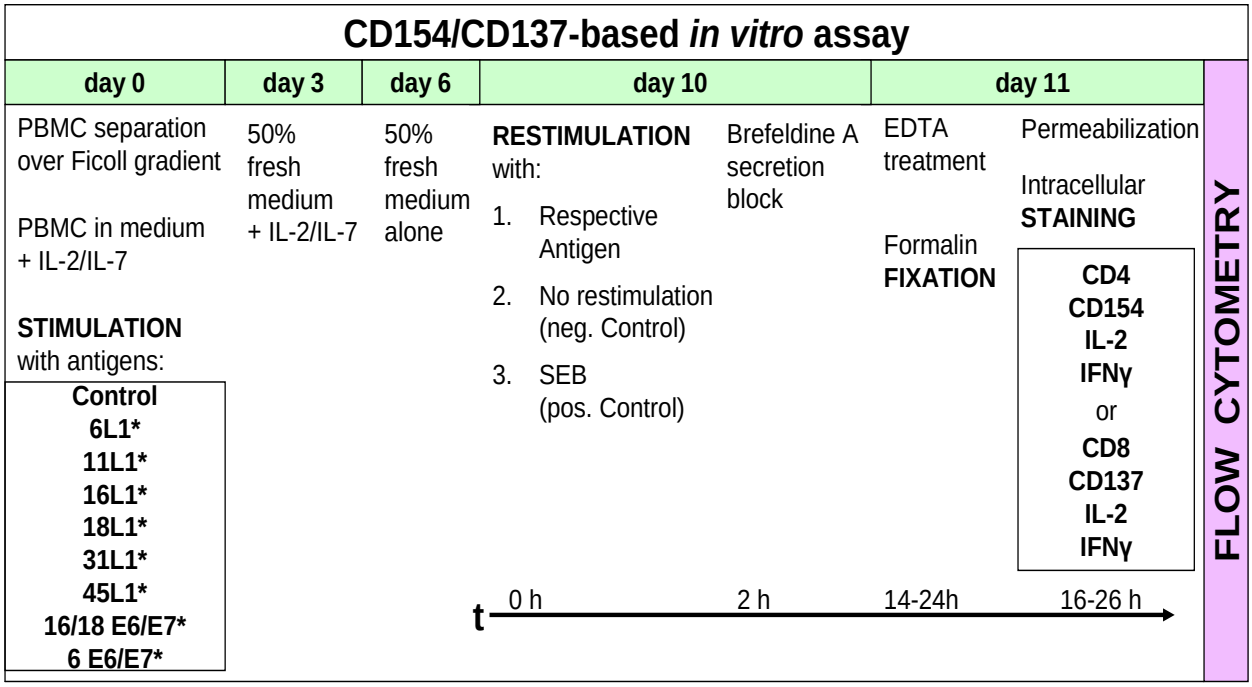

Fig. 8: Schematic representation of the CD154/CD137-based *in vitro* assays

CD: cluster of differentiation; **PBMC:** peripheral blood mononuclear cells; **IL-2/7:** interleukin-2/7; **L1:** late protein 1; **E6/E7:** early protein 6/7; **SEB:** staphylococcal enterotoxin B; **EDTA:** ethylenediaminetetraacetic acid; **IFNγ:** interferon gamma;

* human papillomavirus peptide pools.

2.5 Flow cytometry

2.5.1 Introduction to flow cytometry

Flow cytometry (see Fig. 9) is a technique for the analysis of multiple parameters of individual cells within a heterogeneous population. In order to perform this analysis the flow cytometer passes thousands of cells per second through a laser beam and captures the light that emerges from each cell as it passes through. For accurate data collection it is important that the particles are passed through the laser beam one at a time. This is achieved by hydrodynamic focusing of the cells in a stream of fluid.

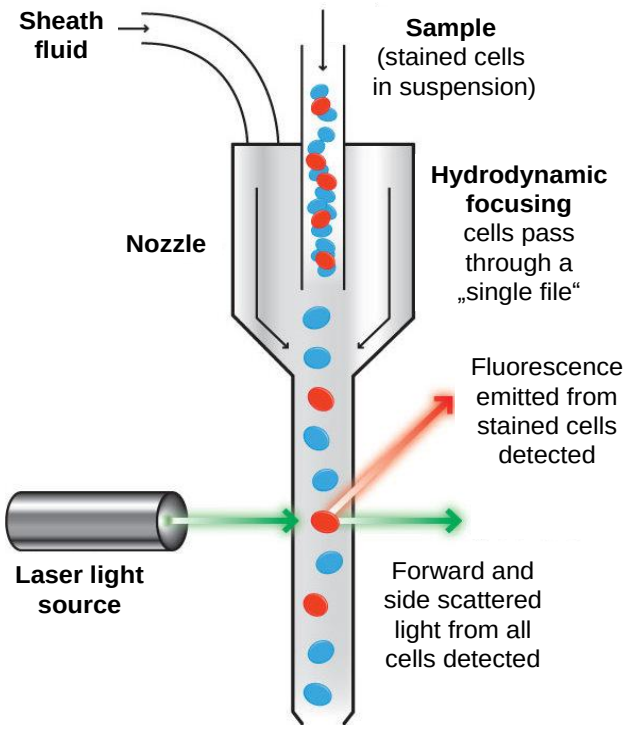

Fig. 9: Flow cytometry [90]

Depending on their size and granularity, cells scatter light at different angles. The magnitude of forward scatter (FSC) is roughly proportional to the size of the cell, whereas the side scatter (SSC) is dependent on the granularity and structural complexity within the cell. In addition, fluorescence labelled antibodies to specific surface or intracellular proteins can be used to mark the cells. Once passed through the laser beam, fluorescent particles may be excited into emitting light at different wavelengths. This combination of scattered and fluorescent light is picked up by the detectors. Analysis of the fluctuations in brightness at each detector makes it possible to derive various types of information about the physical and chemical structure of each individual cell and enables the set parameters to be quantified. Due to the increasing number of lasers and fluorescence detectors, modern devices allow for multiple antibody labelling and are able to identify a target population more precisely by their phenotypic markers.[90,91]

2.5.2 Acquisition of data

In this study the acquisition of all flow cytometric data was done using the FACS Calibur device from BD, which has four different fluorescence detectors. The use of the FSC and SSC enabled us to identify viable lymphocytes within a heterogeneous cell population. These were

subsequently gated into region 1 (R1). The detection of lymphocytes that were positive for CD4, CD154, IFNγ and IL-2 or IL-4, as well as the lymphocytes positive for CD8, CD137, IFNγ and IL-2, occurred on the basis of fluorescent signals emitted by the conjugated antibodies. During the data acquisition, CD4+ or CD8+ T cells out of all viable lymphocytes were additionally gated into R2. The goal was to acquire 200 000 CD4+ T cells per sample in the *ex vivo* settings, though this was not always achieved due to the low number of cells in some samples. In the *in vitro* settings, our goal was to acquire approximately 20 000 CD4+ or CD8+ T cells per sample, although again we were often unable to achieve this, especially when samples used to detect the CD8+ T cells were concerned.

2.5.3 Data analysis

For the analysis of the flow cytometric data we used the CellQuest Pro Software form BD. This program represented the detected characteristics of the cells on various dot plots which were subsequently used for the analysis. The use of the gating function enabled us to subgroup and quantify the cells according to their specific characteristics.

We were interested in quantifying the amount of lymphocytes that were positive for CD4 and CD154 or CD8 and CD137, and at the same time positive for IFNγ, IL-2 or IL-4. In order to achieve this, the two gates described earlier, R1 and R2, were used to filter out viable CD4+ or CD8+ T cells from all the cells detected by the flow cytometer. These were then represented on various dot plots which were used to analyse the presence of CD154, IFNγ, IL-2 and IL-4 positive CD4 T cells or CD137, IFNγ and IL-2 positive CD8 T cells. The y-axis of the dot plot represented the signal intensity of CD154 or CD137, and the x-axis the signal intensity of IFNγ, IL-2 or IL-4. Each dot plot was separated into four quadrants, which were adjusted to the negative control. The cells in the upper right quadrant were regarded as the antigen-specific CD4 or CD8 T cells, expressing the activation marker (CD154 or CD137) and producing in addition either IFNγ, IL-2 or IL-4, the "double positive cells" (see Fig. 10).

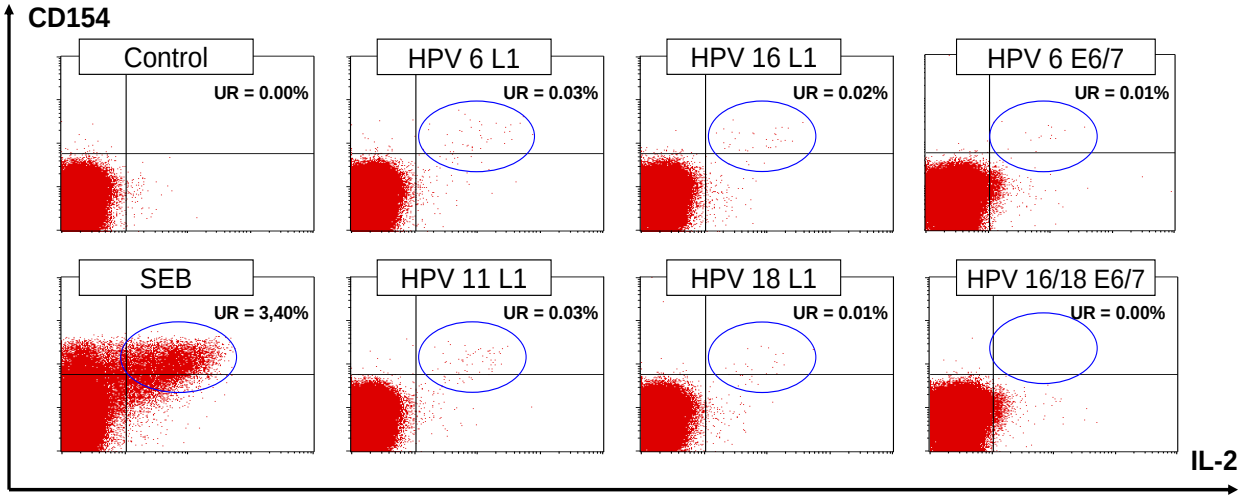

Fig. 10: Dot-plots of the CD4+ T cells detected using the flow-cytometry

The figure shows multiple dot-plots of the CD4+ T cells detected following the *ex vivo* stimulation of whole-blood from one representative Gardasil® vaccinated subject. The CD4+ T cells in the upper right quadrant of each dot plot represent the antigen-specific CD4+ T cells, expressing both CD154 and IL-2 ("double positive cells").

CD: cluster of differentiation; **HPV:** human papillomavirus; **L1:** late protein 1; **E6/E7:** early protein 6/7; **SEB:** staphylococcal enterotoxin B; **IL-2:** interleukin-2; **UR:** upper right quadrant.

2.6 Serology

Serological measurements of the HPV-specific antibody titres were carried out in cooperation with the study group from Dr. M. Pawlita at the "Deutsches Krebsforschungszentrum" (DKFZ) in Heidelberg. Following the blood sampling, serum samples from 33 out of 34 subjects included in the study were collected, stored at -20°C and later sent by the overnight-express to Heidelberg. There, the detection of the antibodies specific for the L1 protein of HPV 6, 11, 16, 18 and 31 was performed using the VLP-based capture ELISA assay established by Peng et al. 1999.[92]

First, each well on the ELISA plates was coated with a solution consisting of 5 µg of a polyclonal serum diluted in 50 µl of PBS. The polyclonal sera were won from the rats which were previously immunised with the VLPs of the respective HPV types. Following the incubation overnight at 4°C, the coating solution was removed and the coated ELISA plates were washed four times with the washing buffer consisting of PBS and 0.05% (w/v) Tween 20. Coated ELISA plates were then incubated for one hour at 37°C with 200 µl of a blocking solution per well. The blocking solution consisted of PBS, 0.05% (w/v) Tween 20 and 5% (w/v) skim milk powder. After the blocking solution was removed, 0.5 µg of the VLPs of the respective HPV type was diluted in 50 µl of the blocking solution and added to each well on half of the coated ELISA plates. The other plates served as negative controls and their wells were supplemented with 50 µl of blocking solution only. All plates were incubated for one hour at

37°C and washed four times. The wells in the first row of all ELISA plates were then supplemented with 100 µl of subjects sera diluted 1:100 with the blocking solution. Next, 50 µl of this solution was transferred into the second row and diluted with further 50 µl of the blocking solution. Such diluting steps were performed altogether 10 times. In this manner 1:2 titration was achieved. Following the one hour incubation at 37°C, the ELISA plates were washed four times and each well was subsequently supplemented with 50 µl of Donkey-Anti-Human-IgG-Peroxidase antibodies. Again the plates were incubated for one hour at 37°C and washed four times. The wells were then supplemented with 100 µl of ABTS each and the absorbance was read at 405 nm wavelength using the Multiplate Reader Multiskan EX (Thermo Fischer Scientific, Waltham, USA) after 15 and 30 minutes. The antibody titer was defined as the greatest dilution for which the difference between the absorbance of the sample with and the one without added VLPs was higher than 0.1. The limit value of 0.1 was chosen randomly and the antibody titer measurements were expressed as median values.

2.7 Questionnaire

At the study enrolment each woman was asked to fill out a pre-formulated questionnaire used to closer define the study participants and verify the homogeneity of the study collective. The completion of the questionnaire was optional and the questions considered too intimate needed not to be answered. The questionnaire was divided into three sections, each consisting of three to five multiple choice questions.

The first section dealt with questions about existing allergy, known immunosuppressive conditions and smoking habits, with the goal to identify possible immunomodulating factors that could lead to alterations of the vaccine-induced cellular immune response. Allergic reactions could cause activation of high numbers of unspecific T lymphocytes, whereas immunosuppressive conditions and chronic nicotine exposure could result in the inability of the subjects' immune system to develop the HPV-specific cellular immune response.[80,93,94]

The second section was used to determine the risk of a possible prior HPV infection. Women were asked questions about their current sexual behaviour, number of lifetime sexual partners, age at sexual debut and condom utilisation. Especially the number of lifetime sexual partners is considered to be the major risk factor for the acquisition of the HPV infection.[15,17]

In the third section women were asked whether they undergo regular gynaecologic screening, whether pathological Pap smears have been detected, an HPV infection diagnosed or they

suffered from condylomata acuminata in the past, and whether there was a history of other sexually transmitted diseases.

2.8 Statistical analysis

The statistical analysis of the data collected was performed using the PASW Statistics 18 software for Windows (SPSS Inc., Chicago, USA).

To summarize the data collected, descriptive statistics, including the mean and the median as the estimates of the central tendency as well as the standard deviation and the interquartile range as the measures of dispersion, were used and the data were represented in the form of tables, box-plots and scatter-plots.

In order to verify the comparability of the two study groups (Gardasil® and Cervarix™ groups), the age of the study participants, the time between the completion of the full vaccination cycle and blood sampling as well as the data collected using the pre-formulated questionnaire were analysed. The data set regarding the age of the study participants and the data set regarding the time between the completion of the full vaccination cycle and blood sampling were tested for normality of the distribution using the one-sample Kolmogorov-Smirnov test. Since the null hypothesis (H_0: The data set follows a normal distribution) was rejected for both data sets, nonparametric Mann-Whitney-U test for two independent samples was subsequently performed in order to test whether the distributions of the two study groups were equal. The immunization with one of the two vaccines (Gardasil® vs. Cervarix™) represented the grouping variable, whereas the age of the study participants or the time between the completion of the full vaccination cycle and blood sampling represented the test variable. In order to allow for the analysis of the data collected using the questionnaire, possible answers were coded with numbers 1 to 4 and subsequently cross tabulated. For crosstabs with two rows (Gardasil® or Cervarix™) and two columns (e.g. allergy: yes or no), Fisher's exact test was performed, whereas for crosstabs with two rows and more than two columns (e.g. condom utilisation: always, occasionally or never), Pearson's chi-square test was performed to test the significance of the deviation from the null hypothesis (H_0: There is no difference between the two groups).

Prior to the analysis of the T cell and antibody data, the data sets were tested for normality of the distribution using once again the nonparametric one-sample Kolmogorov-Smirnov test. Normally distributed data sets were subsequently analysed with the help of the Student's t-test for independent samples, whereas the data sets for which the null hypothesis (H_0: The data set

follows a normal distribution) was rejected were analysed using the nonparametric Mann-Whitney-U test for two independent samples.

In order to account for the differences in the T cell response and the serological response to various HPV types, the T cell data collected using the *ex vivo* and *in vitro* assay as well as the serological data were first analysed within each group separately. For both the Student's t-test and the Mann-Whitney-U test, the stimulation with one of the HPV peptide pools represented the grouping variable, whereas the respective antigen-specific T cell frequency or antibody titer represented the test variable. Subsequently, the Student's t-test and the Mann-Whitney-U test were conducted to analyse the differences between the T cell responses and the serological responses induced by the two vaccines. For this analysis, the immunization with one of the two vaccines (Gardasil® vs. Cervarix™) represented the grouping variable, whereas various antigen-specific T cell frequencies, the antigen-specific expansion indices or the antigen-specific antibody titers represented the test variable.

Due to the small sample size and great dispersion of the data collected using the *in vitro* assay, the one-sample Kolmogorov-Smirnov test was not performed and the distribution was assumed to be not normal. In order to increase the statistical power by increasing the sample size, we pooled the *in vitro* data into low-risk (HPV 6 and 11 L1), high-risk (HPV 16 and 18 L1), cross-reactive (HPV 31 and 45 L1) and natural infection control groups (HPV 6 and 16/18 E6/E7), and performed additional analysis on the pooled data (see Chapter 3.3).

In order to measure the strength of the monotonic relationship between the antigen-specific T cell response and the antigen-specific serological response, the Spearman's rank correlation coefficient (Spearman's rho) was calculated. The Spearman's rho was chosen as the appropriate bivariate correlation procedure for the following reasons: 1.) A linear relationship between the T cell responses and the serological responses was not indicated by the scatter-plots of the respective data; 2.) The Spearman's rho is less sensitive than the Pearson's correlation coefficient to strong outliers that were found in the tails of both data sets. For formation of the scatter-plots and calculation of the Spearman's rho, only the frequencies of the antigen-specific IL-2-producing T cells were used, as the role of the IFNγ-producing Th1 cells in the induction of the humoral immune responses is minor (see Chapter 1.4.2).

Since the sample size of the data sets in the present study is generally low, the level of significance for all applied statistical tests was chosen to be 0.05. Taking into account that the deviations from the null hypothesis can occur in both directions, two-tailed p-values were

calculated. For the nonparametric tests used (Kolmogorov-Smirnov, Mann-Whitney-U test and Spearman's rho) exact two-tailed p-values were calculated. The confidence interval from the previously described Student's t-test was chosen to be 95%.

Finally it is important to mention that the analysis of the T cell data was performed separately for each cytokine.

3 RESULTS

3.1 Characterisation of the study collective

3.1.1 Age distribution

The average age of all 34 women included in the study was 28 years, the youngest women being 21 and the oldest 53 years old. Women immunized with Gardasil® were on average 25 years old with a standard deviation of 3.1 years, whereas women immunized with Cervarix™ were on average 30 years old with a standard deviation of 10.5 years. The difference in age between the two groups was found not to be significant ($p = 0.190$). The average age of 14 women whose blood was used to conduct the *in vitro* assay was 26 years (SD = 2.7) with no significant difference in age between the two groups ($p = 0.054$).

3.1.2 Time between blood sampling and vaccination

All 34 women included in the study completed the full vaccination cycle on average 4.5 years (SD = 1.1) prior to the study involvement. The time between the blood sampling and the completion of the full vaccination cycle for women immunized with Gardasil® or Cervarix™ was on average 5.0 (SD = 1.1) and 4.1 (SD = 1.0) years respectively, with no significant difference being found between the two groups ($p = 0.312$). For the 14 women whose blood was used to conduct the *in vitro* assay, the difference in the time between the blood sampling and the completion of the full vaccination cycle was 5.6 (SD = 0.2) and 3.6 (SD = 0.1) years in the Gardasil® and Cervarix™ groups respectively, with the difference between the two groups being highly significant ($p < 0.001$).

3.1.3 Immunomodulating factors

With respect to allergy, immunosuppressive conditions or smoking habits no significant differences were found between the two groups. One women in each group had a known immunosuppressive disease (see Table 3).

3.1.4 Sexual behaviour

The two groups showed no significant differences with respect to relationship status, number of lifetime sexual partners or age at sexual debut. One woman in the Cervarix™ group was a virgin at the study enrolment, whereas all women in the Gardasil® group have had sexual contact in the past. Significant difference between the two groups was found with respect to condom use. The majority of the women in the Gardasil® group occasionally use a condom, whereas 50% of the women in the Cervarix™ group never use a condom (see Table 3).

3.1.5 Gynaecologic anamnesis

All women in the Gardasil® group underwent regular gynecological screening, whereas two women in the Cervarix™ group did not. This difference between the two groups is not statistically significant.

With respect to detection of pathological Pap smears, HPV infection, condylomata acuminata or other sexually transmitted diseases no significant differences were found between the two groups. Two women in the Gardasil® group reported a pathological Pap smear on one occasion and one further woman reported a prior Pap IIID lesion, which was cured via laser treatment. None of the three women reported a history of known HPV infection. In the Cervarix™ group two women reported a pathological Pap smear on one occasion, one of them reporting a history of a positive HPV test (exact HPV type was unknown). None of the women who completed the questionnaire reported a history of condylomata acuminata or other sexually transmitted diseases (see Table 3).

Table 3: Questionnaire - Characterisation of the study collective

		Gardasil® n=15	Cervarix™ n=16	Significance (p-value)
Immunomodulating factors				
Allergy	▫ No ▫ Yes	10 (67%) 5 (33%)	13 (81%) 3 (19%)	n.s. (0.433)
Immunosuppression	▫ No ▫ Yes	14 (93%) 1 (7%)	15 (94%) 1 (6%)	n.s. (1.000)
Nicotine abuse	▫ No ▫ In the past ▫ Yes	7 (47%) 2 (13%) 6 (40%)	8 (50%) 3 (19%) 5 (31%)	n.s. (1.000)
Sexual behaviour				
Relationship	▫ Stabile relationship ▫ Changing sexual partners ▫ Not sexually active	8 (67%) 1 (8%) 3 (25%)	9 (56%) 2 (13%) 5 (31%)	n.s. (0.866)
Number of lifetime sexual partners	▫ Virgin ▫ ≤ 5 ▫ > 5	0 (0%) 6 (50%) 6 (50%)	1 (6%) 7 (44%) 8 (50%)	n.s. (1.000)
Age at sexual debut	▫ Virgin ▫ < 14 years ▫ 14 to 18 years ▫ > 18	0 (0%) 0 (0%) 9 (75%) 3 (25%)	1 (6%) 1 (6%) 13 (81%) 1 (6%)	n.s. (0.294)
Condom utilisation	▫ Always ▫ Occasionally ▫ Never	4 (33%) 7 (58%) 1 (8%)	6 (38%) 2 (13%) 8 (50%)	* (0.018)
Gynecological anamnesis				
Regular gynaecological screening	▫ No ▫ Yes	0 (0%) 15 (100%)	2 (13%) 14 (88%)	n.s. (0.484)
Pathological Pap smear	▫ No regular Pap smears ▫ No pathological findings ▫ Yes, on one occasion ▫ Yes, further procedures necessary	1 (7%) 11 (73%) 2 (13%) 1 (7%)	3 (19%) 11 (69%) 2 (13%) 0 (0%)	n.s. (0.838)
HPV infection	▫ No ▫ Yes, in the past ▫ Yes, at the moment	15 (100%) 0 (0%) 0 (0%)	15 (94%) 1 (6%) 0 (0%)	n.s. (0.516)
Condylomata acuminata	▫ No ▫ Yes, in the past ▫ Yes, at the moment	15 (100%) 0 (0%) 0 (0%)	16 (100%) 0 (0%) 0 (0%)	No difference
Other sexually transmitted diseases	▫ No ▫ Yes	15 (100%) 0 (0%)	16 (100%) 0 (0%)	No difference

n: number of subjects; **n.s.:** not significant; ***:** $p < 0.05$; **Pap smear:** cytological examination of the cervical smear according to Papanicolaou; **HPV:** human papillomavirus.

3.2 Results of the ex vivo assay

3.2.1 Definition of limits and exclusion criteria (Analysis approach)

For every sample analysed using the *ex vivo* assay, the percentage of the activated CD4+/CD154+ T cells and the percentage of specifically activated CD4+/CD154+ T cells which produced IFNγ, IL-2 or IL-4 (double positive cells) among all CD4+ T cells was determined. In order to preserve the clarity of the study, only the latter are presented. Years after the vaccination these double positive cells represent the short term activated memory T cells.

For the negative control, i.e. unstimulated blood sample, the percentage of the antigen-specific cells, either CD4+/CD154+/IFNγ+ or CD4+/CD154+/IL-2+ or CD4+/CD154+/IL-4+ T cells, among all CD4+ T cells was not allowed to exceed 0.04%. This criterion was satisfied for all subjects enrolled. In most of the cases, the frequency of the antigen-specific cells in the negative control was 0.00%, as expected. Several subjects showed frequencies of up to 0.04% in the negative control. In order to account for this background stimulation, we decided to subtract the results of the negative control from all antigen stimulations of the respective subject. These corrected results were subsequently used for the statistical analysis. Of note is that the frequencies of the antigen-specific cells in the antigen stimulations of the respective subjects were almost always higher than, and occasionally equal to, the frequencies in the negative control.

The frequency of the antigen-specific cells detected in the samples stimulated with SEB (positive control) needed to be higher than 0.5% in order to consider the cells of the respective subject as viable. This condition was not satisfied in two cases. Thus, one woman from the Gardasil® and one from the Cervarix™ group with SEB controls lower than 0.5% were excluded from further analysis (see Fig. 6).

Due to IT problems analysis of several antigen stimulations from various subjects were lost (e.g. stimulation with L1 peptide pool of HPV 11 from one subject from the Cervarix™ group). This did not result in the exclusion of the respective subjects, as the antigen stimulations are independent of each other.

In order to analyse the cells by flow cytometry two gates needed to be set, the lymphocyte gate, “R1”, and the CD4+ T cell gate, “R2” (see Chapter 2.5.2). The use of these two gates is crucial for calculating the frequencies of the detected antigen-specific cells. For four women from the Cervarix™ group, a distinction between the CD4+ and CD4- T cell populations was not

possible. In order to avoid the falsification of the calculated frequencies, all four women were excluded from the study.

3.2.2 Analysis of T cell frequencies within the two groups

3.2.2.1 Overview

As previously mentioned, only 34 from 40 women recruited were included in the analysis. Following the *ex vivo* stimulation, HPV L1-specific T helper cells were detected in all 34 women immunized with either Garadsil® or Cervarix™ on average 4.5 years prior to the study enrolment.

For all 34 women the frequency of the detected CD4+/CD154+/IFNγ+, CD4+/CD154+/IL-2+ or CD4+/CD154+/IL-4+ T cells in the negative control was made to be a constant at 0.00% (see Chapter 3.2.1). The SEB control was always higher than 0.5%, ranging from 0.54 to 24.22%. The mean frequency of the CD4+/CD154+/IFNγ+ T cells stimulated by SEB in the Garadasil® and the Cervarix™ group was 3.383% (SD = 1.850) and 4.771% (SD = 3.133), respectively, whereas the mean frequency of the CD4+/CD154+/IL-2+ T cells was 10.207% (SD = 5.017) and 11.873% (SD = 6.957), respectively. IL-4-producing T cells stimulated by SEB were not analysed, as the viability of the cells was confirmed by the detectable IFNγ- and IL-2-producing T cells.

3.2.2.2 Gardasil®

On average 5.0 years (SD = 1.1) after the completion of the full vaccination cycle consisting of three doses of Gardasil® the mean frequency of the detected CD4+/CD154+/IFNγ+ T cells specific for the L1 protein from HPV 6, 11, 16 and 18 was 0.024%, 0.023%, 0.026% and 0.008%, respectively. The HPV 6-, 11- and 16-specific IFNγ-producing T cells were detected at comparable frequencies, whereas the frequency of the HPV 18-specific IFNγ-producing T cells was on average three times lower. These differences were statistically significant (see Table 4).

Table 4: Gardasil® - Mean frequency of the antigen-specific CD4+/CD154+/IFNγ+ T cells

GARDASIL® IFNγ		HPV 6 L1	HPV 11 L1	HPV 16 L1	HPV 18 L1
Frequency of CD4+/CD154+/IFNγ+ T cells	mean (±SD)	**0.024** (±0.018)	**0.023** (±0.021)	**0.026** (±0.036)	**0.008** (±0.018)
HPV 6 L1					
HPV 11 L1		n.s.			
HPV 16 L1		n.s.	n.s.		
HPV 18 L1		***	**	**	

The table shows the percentage of the antigen-specific CD4+/CD154+/IFNγ+ T cells among all CD4+ T cells. The significance of the differences between the HPV L1 antigens is represented by the p-values: **n.s.** = not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

IFNγ: interferon gamma; **HPV:** human papillomavirus; **L1:** late protein 1; **CD:** cluster of differentiation; **SD:** standard deviation.

Cross-reactive CD4+/CD154+/IFNγ+ T cells were detected in three out of four subjects from the Gardasil® group whose blood was stimulated with L1 peptide pools of HPV 31 and 45. The mean frequencies of the HPV 31- and 45-specific IFNγ-producing T cells were in both cases 0.008% (SD = 0.005). These were comparable to the frequency of the HPV 18-specific IFNγ-producing T cells and were 3.3-times lower than the frequency of the HPV 16-specific IFNγ-producing T cells.

As far as the possible natural infection is concerned, in 8 out of 15 women immunised with Garadasil® CD4+/CD154+/IFNγ+ T cells specific for E6/E7 proteins from HPV 6 were detected, the frequency being on average 0.007% (SD=0.009), whereas no CD4+/CD154+/IFNγ+ T cells specific for E6/E7 protein from HPV 16 and 18 could be detected.

The CD4+/CD154+/IL-2+ T cells specific for the L1 protein from HPV 6, 11, 16 and 18 were found at 1.9- to 3.4-times higher frequencies compared to the frequencies of the CD4+/CD154+/IFNγ+ T cells specific for the respective proteins. The mean frequency of the detected CD4+/CD154+/IL-2+ T cells specific for the L1 protein from HPV 6, 11, 16 and 18 was 0.045%, 0.051%, 0.053% and 0.027%, respectively. The HPV 6-, 11- and 16-specific IL-2-producing T cells were detected at comparable frequencies, whereas the frequency of the HPV 18-specific IL-2-producing T cells was approximately two times lower. These differences were statistically significant (see Table 5).

Table 5: Gardasil® - Mean frequency of the antigen-specific CD4+/CD154+/IL-2+ T cells

GARDASIL® IL-2		HPV 6 L1	HPV 11 L1	HPV 16 L1	HPV 18 L1
Frequency of CD4+/CD154+/IL-2+ T cells	mean (±SD)	**0.045** (±0.028)	**0.051** (±0.032)	**0.053** (±0.060)	**0.027** (±0.032)
HPV 6 L1					
HPV 11 L1		n.s.			
HPV 16 L1		n.s.	n.s.		
HPV 18 L1		**	**	*	

The table shows the percentage of the antigen-specific CD4+/CD154+/IL-2+ T cells among all CD4+ T cells. The significance of the differences between the HPV L1 antigens is represented by the p-values: **n.s.** = not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

IL-2: interleukin-2; **HPV:** human papillomavirus; **L1:** late protein 1; **CD:** cluster of differentiation; **SD:** standard deviation.

Cross-reactive CD4+/CD154+/IL-2+ T cells were detected in all four subjects from the Gardasil® group whose blood was stimulated with L1 peptide pools of HPV 31 and 45. The mean frequency of the HPV 31- and 45-specific IL-2-producing T cells was 0.030% (SD = 0.022) and 0.015% (SD = 0.010), respectively. Comparing the frequencies of the IL-2-producing T cells specific for HPV 16 with those specific for HPV 31, approximately two times higher values are detected for the vaccine type HPV. The same is true when comparing the IL-2-producing T cells specific for HPV 18 with those specific for HPV 45.

As far as the possible natural infection is concerned, in 13 out of 15 women immunised with Garadasil® CD4+/CD154+/IL-2+ T cells specific for E6/E7 proteins from HPV 6 were detected, whereas only four showed specific response to E6/E7 proteins from HPV 16 and 18. The mean frequency of the IL-2-producing T cells specific for E6/E7 proteins from HPV 6 and 16/18 was 0.019% (SD = 0.012) and 0.003% (SD = 0.005), respectively.

Only three out of 11 women immunised with Gardasil® showed an IL-4 positive response to stimulations with L1 peptide pools of HPV 6, 11, 16 and 18. The mean frequencies of the detected CD4+/CD154+/IL-4+ T cells specific for the vaccine type HPV were lower than 0.01% and did not differ significantly from the negative control (see Chapter 3.2.3.3).

3.2.2.3 Cervarix™

On average 4.1 years (SD = 1.0) after the completion of the full vaccination cycle consisting of three doses of Cervarix™ the mean frequency of the detected CD4+/CD154+/IFNγ+ T cells specific for the L1 protein from HPV 6, 11, 16 and 18 was 0.016%, 0.012%, 0.020% and 0.011%, respectively. The strongest IFNγ response was induced by the stimulation with L1

peptide pool from HPV 16, being 1.8-times higher than the response induced by L1 peptide pool from HPV 18. This difference in response was statistically significant (see Table 6).

Table 6: Cervarix™ - Mean frequency of the antigen-specific CD4+/CD154+/ IFNγ+ T cells

CERVARIX™ IFNγ		HPV 6 L1	HPV 11 L1	HPV 16 L1	HPV 18 L1
Frequency of CD4+/CD154+/IFNγ+ T cells	mean (±SD)	**0.016** (±0.021)	**0.012** (±0.009)	**0.020** (±0.019)	**0.011** (±0.010)
HPV 6 L1					
HPV 11 L1		n.s.			
HPV 16 L1		n.s.	n.s.		
HPV 18 L1		n.s.	n.s.	*	

The table shows the percentage of the antigen-specific CD4+/CD154+/IFNγ+ T cells among all CD4+ T cells. The significance of the differences between the HPV L1 antigens is represented by the p-values: **n.s.** = not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

IFNγ: interferon gamma; **HPV:** human papillomavirus; **L1:** late protein 1; **CD:** cluster of differentiation; **SD:** standard deviation.

Cross-reactive CD4+/CD154+/IFNγ+ T cells were detected in 4 out of 6 subjects from the Cervarix™ group whose blood was stimulated with L1 peptide pools of HPV 31 and 45. The mean frequency of the HPV 31- and 45-specific IFNγ-producing T cells was 0.008% (SD = 0.008) and 0.010% (SD = 0.012), respectively. These were comparable to the frequency of the HPV 18-specific IFNγ-producing T cells and were approximately two times lower than the frequency of the HPV 16-specific IFNγ-producing T cells.

CD4+/CD154+/IFNγ+ T cells specific for E6/E7 proteins from HPV 6 were detected in 4 out of 19 women immunized with Cervarix™, whereas only one woman showed an IFNγ response to E6/E7 proteins from HPV 16/18. The mean frequency of the detected IFNγ-producing T cells specific for E6/E7 proteins from HPV 6 and HPV 16/18 was 0.003% (SD = 0.006) and 0.001% (SD = 0.002), respectively.

Compared to the frequencies of the CD4+/CD154+/IFNγ+ T cells specific for the L1 protein from HPV 6, 11, 16 and 18, the CD4+/CD154+/IL-2+ T cells specific for the respective proteins were found at 2.8- to 3.4-times higher frequencies. The mean frequency of the detected CD4+/CD154+/IL-2+ T cells specific for the L1 protein from HPV 6, 11, 16 and 18 was 0.045%, 0.033%, 0.058% and 0.037%, respectively. The strongest IL-2 response was induced by the stimulation with L1 peptide pool from HPV 16, being 1.6-times higher than the response induced by L1 peptide pool from HPV 18. This difference in response was statistically not significant (see Table 7).

Table 7: Cervarix™ - Mean frequency of the antigen-specific CD4+/CD154+/IL-2+ T cells

CERVARIX™ IL-2		HPV 6 L1	HPV 11 L1	HPV 16 L1	HPV 18 L1
Frequency of CD4+/CD154+/IL-2+ T cells	mean (±SD)	**0.045** (±0.065)	**0.033** (±0.020)	**0.058** (±0.068)	**0.037** (±0.047)
HPV 6 L1					
HPV 11 L1		n.s.			
HPV 16 L1		n.s.	n.s.		
HPV 18 L1		n.s.	n.s.	n.s.	

The table shows the percentage of the antigen-specific CD4+/CD154+/IL-2+ T cells among all CD4+ T cells. The significance of the differences between the HPV L1 antigens is represented by the p-values: **n.s.** = not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

IL-2: interleukin-2; **HPV:** human papillomavirus; **L1:** late protein 1; **CD:** cluster of differentiation; **SD:** standard deviation.

Cross-reactive CD4+/CD154+/IL-2+ T cells were detected in four out of five and all five subjects from the Cervarix™ group whose blood was stimulated with L1 peptide pools of HPV 31 and 45, respectively. The mean frequency of the HPV 31- and 45-specific IL-2-producing T cells was 0.016% (SD = 0.013) and 0.036% (SD = 0.036), respectively. The IL-2 response induced by stimulation with L1 peptide pool from HPV 16 was 3.6-times stronger than the response induced by stimulation with L1 peptide pool from the HPV 31, whereas the IL-2 responses induced by stimulations with L1 peptide pools from HPV 18 and 45 were detected at comparable levels.

CD4+/CD154+/IL-2+ T cells specific for E6/E7 proteins from HPV 6 were detected in 12 women from the Cervarix™ group, whereas only 5 women showed specific response to E6/E7 proteins from HPV 16/18. The mean frequency of the IL-2-producing T cells specific for E6/E7 proteins from HPV 6 and 16/18 was 0.008% (SD = 0.008) and 0.003% (SD = 0.005), respectively.

Only three out of 13 women immunised with Cervarix™ showed an IL-4 positive response to stimulations with L1 peptide pools of HPV 6, 11, 16 and 18. The mean frequencies of the detected CD4+/CD154+/IL-4+ T cells specific for the vaccine type HPV were lower than 0.01% and did not differ significantly from the negative control (see Chapter 3.2.3.3).

3.2.3 Comparison of the T cell frequencies (Gardasil® vs. Cervarix™)

3.2.3.1 Interferon-γ

Table 8 shows the mean frequencies of the antigen-specific CD4+/CD154+/IFNγ+ T cells in the Gardasil® and the Cervarix™ group. The significance of the difference is shown by the p-

values. In addition Figure 11 visualises the differences in the frequencies of the antigen-specific IFNγ-producing T cells between the Gardasil® and the Cervarix™ group, showing also the percentage of women in each group with detectable antigen-specific T cell response.

Table 8: Mean frequency of the antigen-specific CD4+/CD154+/IFNγ+ T cells in women immunized with either Gardasil® or Cervarix™

Antigen	GARDASIL®		CERVARIX™		Significance (p-value)
	CD4/CD154/IFNγ positive T helper cells Mean frequency (± SD)	No. of subjects analysed	**CD4/CD154/IFNγ positive T helper cells** Mean frequency (± SD)	No. of subjects analysed	
Co.	0 (± 0.000)	15	0 (± 0.000)	19	Variable is a constant
SEB	3.383 (± 1.850)	15	4.771 (± 3.133)	19	n.s. (0.139) †
HPV 6 L1	0.024 (± 0.018)	15	0.016 (± 0.021)	19	n.s. (0.051) ‡
HPV 11 L1	0.023 (± 0.021)	15	0.012 (± 0.009)	18	n.s. (0.075) ‡
HPV 16 L1	0.026 (± 0.036)	15	0.020 (± 0.019)	19	n.s. (0.831) ‡
HPV 18 L1	0.008 (± 0.018)	15	0.011 (± 0.010)	19	n.s. (0.054) ‡
HPV 31 L1	0.008 (± 0.005)	4	0.008 (± 0.008)	5	n.s. (0.920) †
HPV 45 L1	0.008 (± 0.005)	4	0.010 (± 0.012)	5	n.s. (0.716) †
HPV 6 E6/E7	0.007 (± 0.009)	15	0.003 (± 0.006)	18	n.s. (0.084) ‡
HPV 16/18 E6/E7	0 (± 0.000)	15	0.001 (± 0.002)	19	n.s. (1.000) ‡

The table shows the percentage of the antigen-specific CD4+/CD154+/ INFγ+ T cells among all CD4+ T cells. The significance of the differences between the Gardasil® and Cervarix™ group is represented by the p-values: **n.s.** = not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

CD: cluster of differentiation; **IFNγ:** interferon gamma; **Co.:** negative control; **SEB:** staphylococcal enterotoxin B; **HPV:** human papillomavirus; **L1:** late protein 1; **E6/E7:** early proteins E6/E7; **SD:** standard deviation;

† Statistical analysis was done using the independent sample T test;
‡ Statistical analysis was done using the Mann Whitney Test for independent samples.

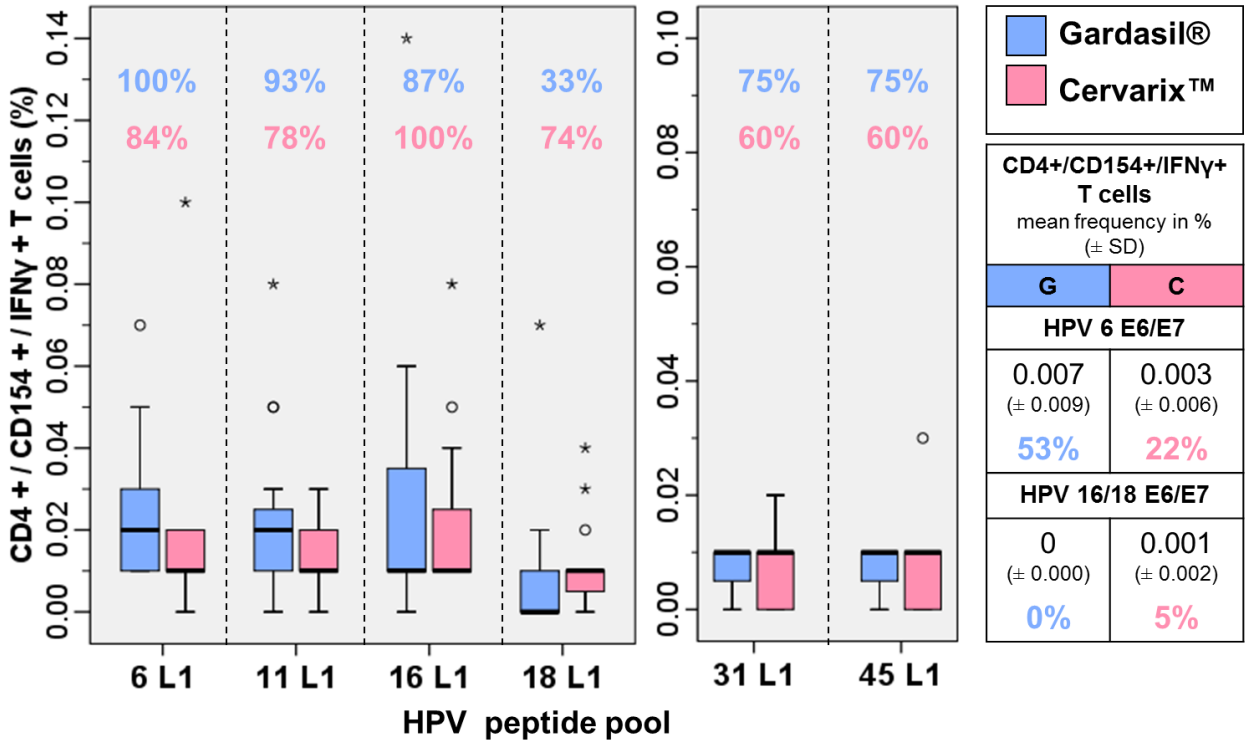

Fig. 11: Comparison of the antigen-specific CD4+/CD154+/IFNγ+ T cell frequencies between the Gardasil® and the Cervarix™ group

The figure shows the percentage of the antigen-specific CD4+/CD154+/INFγ+ T cells among all CD4+ T cells in women immunized with either Gardasil® or Cervarix™. The box represents the middle 50% of the data sample and the line in the box represents the median. The whiskers stretch both sides to maximum 1.5-times the interquartile range (IQR). Mild outliers up to 3-times the IQR are represented by small circles, whereas extreme outliers greater than 3-times the IQR are represented by stars. The small table on the right shows in addition the mean frequencies of the HPV 6 and 16/18 E6/E7-specific CD4+/CD154+/INFγ+ T cells among all CD4+ T cells.

Coloured numbers: percentage of women in each group with detectable antigen-specific T cell response;

CD: cluster of differentiation; **IFNγ:** interferon gamma; **SD:** standard deviation; **G:** Gardasil®; **C:** Cervarix™; **HPV:** human papillomavirus; **E6/E7:** early proteins E6/E7; **L1:** late protein 1.

As previously mentioned, for all 34 women the frequency of the detected CD4+/CD154+/IFNγ+ in the negative control was made to be a constant at 0.00% (see Chapter 3.2.1). The IFNγ response to SEB was 1.4-times stronger in women immunized with Cervarix™ compared to the response measured in women immunized with Gardasil®. This difference was statistically not significant.

The frequency of the detected IFNγ-producing T cells specific for HPV 16 was 1.3-times higher in the Gardasil® vaccinees, whereas the Cervarix™ vaccinees showed 1.4-times higher frequency of the detected IFNγ-producing T cells specific for HPV 18. The differences in the responses to the two high-risk HPV types between the Gardasil® and Cervarix™ group were statistically not significant.

Surprisingly, IFNγ-producing T cells specific for the two low-risk HPV types (HPV 6 and 11) were also detected in women immunized with Cervarix™, although these antigens are not contained in the vaccine. The mean frequency of the IFNγ-producing T cells specific for HPV 6 and 11 induced by Cervarix™ was 0.016% and 0.012%, respectively. The IFNγ responses specific for HPV 6 and 11 induced by Gardasil® were 1.5- and 1.9-times stronger, respectively, the mean frequencies being 0.024% for HPV 6 and 0.023% for HPV 11. Again, statistical analysis showed that the differences in the responses to the two low-risk HPV types between the Gardasil® and Cervarix™ group were not significant.

Both Gardasil® and Cervarix™ induced cross-reactive CD4+/CD154+/IFNγ+ T cells specific for HPV 31 and 45. The mean frequency of the IFNγ-producing T cells specific for HPV 31 in both groups was 0.008%, whereas Cervarix™ vaccinees showed with mean frequency of 0.010% a 1.3-times stronger response to HPV 45. This difference was statistically not significant.

The IFNγ response specific for the E6/E7 peptides from HPV 6 was detected in 53% and 22% of the women immunised with Gardasil® and Cervarix™, respectively, whereas none of the women from the Gardasil® and only one woman from the Cervarix™ group showed specific response to E6/E7 peptides from HPV 16/18. Statistical analysis showed that differences in the mean frequencies of the detected CD4+/CD154+/IFNγ+ T cells specific for E6/E7 peptides from HPV 6 and 16/18 were not significant.

3.2.3.2 Interleukin-2

The CD4+/CD154+/IL-2+ T cells were detected *ex vivo* at higher frequencies than the CD4+/CD154+/IFNγ+ T cells. Table 9 shows mean frequencies of the antigen-specific CD4+/CD154+/IL-2+ T cells in the Gardasil® and the Cervarix™ group. The significance of the difference is shown by the p-values. In addition, Figure 12 visualises the differences in the frequencies of the antigen-specific IL-2-producing T cells between the Gardasil® and the Cervarix™ group, showing also the percentage of women in each group with detectable antigen-specific T cell response.

Table 9: Mean frequency of the antigen-specific CD4+/CD154+/IL-2+ T cells in women immunized with either Gardasil® or Cervarix™

Antigen	GARDASIL®		CERVARIX™		Significance (p-value)
	CD4/CD154/IL-2 positive T helper cells Mean frequency (± SD)	No. of subjects analysed	**CD4/CD154/IL-2 positive T helper cells** mean frequency (± SD)	No. of subjects analysed	
Co.	0 (± 0.000)	15	0 (± 0.000)	19	Variable is a constant
SEB	10.207 (± 5.017)	15	11.873 (± 6.957)	19	n.s. (0.441) †
HPV 6 L1	0.045 (± 0.028)	15	0.045 (± 0.065)	19	n.s. (0.218) ‡
HPV 11 L1	0.051 (± 0.032)	15	0.033 (± 0.020)	18	n.s. (0.052) †
HPV 16 L1	0.053 (± 0.060)	15	0.058 (± 0.068)	19	n.s. (0.735) ‡
HPV 18 L1	0.027 (± 0.032)	15	0.037 (± 0.047)	19	n.s. (0.271) ‡
HPV 31 L1	0.030 (± 0.022)	4	0.016 (± 0.013)	5	n.s. (0.269) †
HPV 45 L1	0.015 (± 0.010)	4	0.036 (± 0.036)	5	n.s. (0.306) †
HPV 6 E6/E7	0.019 (± 0.012)	15	0.008 (± 0.008)	18	** (0.009) ‡
HPV 16/18 E6/E7	0.003 (± 0.005)	15	0.003 (± 0.005)	19	n.s. (1.000) ‡

The table shows the percentage of the antigen-specific CD4+/CD154+/ IL-2+ T cells among all CD4+ T cells. The significance of the differences between the Gardasil® and Cervarix™ group is represented by the p-values: **n.s.** = not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

CD: cluster of differentiation; **IL-2:** interleukin-2; **Co.:** negative control; **SEB:** staphylococcal enterotoxin B; **HPV:** human papillomavirus; **L1:** late protein 1; **E6/E7:** early proteins E6/E7; **SD:** standard deviation;

† Statistical analysis was done using the independent sample T test;
‡ Statistical analysis was done using the Mann Whitney Test for independent samples.

As previously mentioned, for all 34 women the frequency of the detected CD4+/CD154+/IL-2+ T cells in the negative control was made to be a constant at 0.00% (see Chapter 3.2.1). The IL-2 response to SEB was 1.2-times stronger in women immunized with Cervarix™ compared to the response measured in women immunized with Gardasil®. Statistically, the difference was not significant.

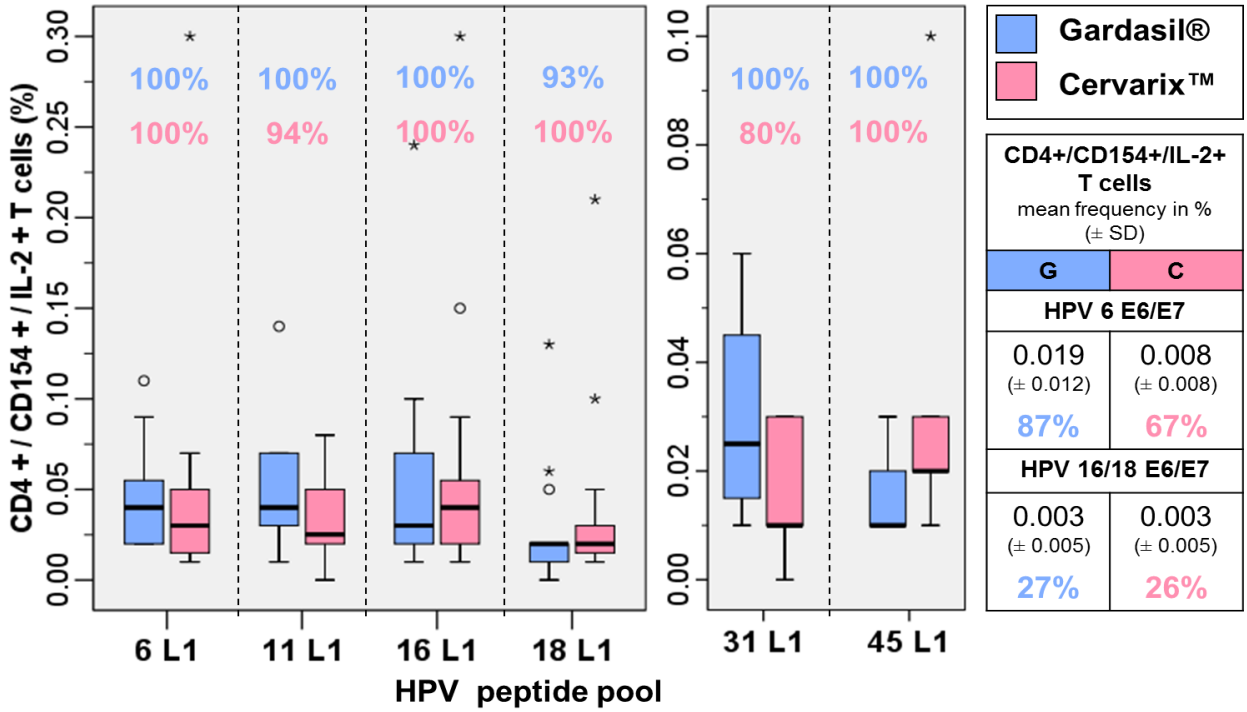

Fig. 12: Comparison of the antigen-specific CD4+/CD154+/IL-2+ T cell frequencies between the Gardasil® and the Cervarix™ group

The figure shows the percentage of the antigen-specific CD4+/CD154+/IL-2+ T cells among all CD4+ T cells in women immunized with either Gardasil® or Cervarix™. The box represents the middle 50% of the data sample and the line in the box represents the median. The whiskers stretch both sides to a maximum of 1.5-times the interquartile range (IQR). Mild outliers up to 3-times the IQR are represented by small circles, whereas extreme outliers greater than 3-times the IQR are represented by stars. The small table on the right shows in addition the mean frequencies of the HPV 6 and 16/18 E6/E7-specific CD4+/CD154+/IL-2+ T cells among all CD4+ T cells.

Coloured numbers: percentage of women in each group with detectable antigen-specific T cell response;

CD: cluster of differentiation; **IL-2:** interleukin-2; **SD:** standard deviation; **G:** Gardasil®; **C:** Cervarix™; **HPV:** human papillomavirus; **E6/E7:** early proteins E6/E7; **L1:** late protein 1.

The mean frequency of the detected CD4+/CD154+/IL-2+ T cells specific for HPV 16 and 18 in the Cervarix™ group was 0.058% and 0.037%, respectively. Compared with the respective frequencies in the Gardasil® group, Cervarix™ showed 1.1-times stronger IL-2 response to HPV 16 and 1.4-times stronger IL-2 response to HPV 18. Statistical analysis showed that these differences in the response to the two high-risk HPV types between the Gardasil® and the Cervarix™ group were not significant.

Taking into account that the VLPs of the low-risk HPV types are not contained in the Cervarix™ vaccine, it was surprising to find that the mean frequency of the detected CD4+/CD154+/IL-2+ T cells specific for HPV 6 was 0.045% in the Gardasil® and also in the Cervarix™ group. The mean frequency of the IL-2-producing T cells specific for HPV 11 in the Gardasil® group was 0.051% and was 1.5-times higher than the respective frequency in the Cervarix™ group (0.033%). This difference was statistically not significant.

Both Gardasil® and Cervarix™ induced cross-reactive CD4+/CD154+/IL-2+ T cells specific for HPV 31 and 45. The mean frequency of the IL-2-producing T cells specific for HPV 31 in the Gardasil® group was 0.030% and was 1.9-times higher than in the Cervarix™ group (0.016%), whereas Cervarix™ with a mean frequency of 0.036% showed a 2.4-times stronger IL-2 response to HPV 45 than Gardasil® (0.015%). These differences in the response to the homologous high-risk HPV types between the two groups were statistically not significant.

The IL-2 response specific for E6/E7 peptides from HPV 6 was detected in 87% and 67% of the women immunised with Gardasil® and Cervarix™, respectively. The mean frequency of the CD4+/CD154+/IL-2+ T cells specific for E6/E7 peptides from HPV 6 was 0.019% in the Gardasil® group and was 2.4-times higher than in the Cervarix™ group (0.008%). This difference was statistically significant (p = 0.009). Only 27% and 26% of the women from the Gardasil® and the Cervarix™ group, respectively, showed specific responses to E6/E7 peptides from HPV 16/18, the mean frequency being 0.003% in both groups.

3.2.3.3 Interleukin-4

Table 10 shows the mean frequencies of the antigen-specific CD4+/CD154+/IL-4+ T cells.

Table 10: Mean frequency of the antigen-specific CD4+/CD154+/IL-4+ T cells in women immunized with either Gardasil® or Cervarix™

Antigen	GARDASIL®		CERVARIX™		Significance (p-value)
	CD4/CD154/IL-4 positive T helper cells Mean frequency (± SD)	No. of subjects analysed	**CD4/CD154/IL-4 positive T helper cells** mean frequency (± SD)	No. of subjects analysed	
Co.	0 (± 0.000)	11	0 (± 0.000)	14	No difference
HPV 6 L1	0.005 (± 0.009)	11	0 (± 0.000)	14	n.s. (0.072) ‡
HPV 11 L1	0.005 (± 0.009)	11	0.001 (± 0.004)	14	n.s. (0.510) ‡
HPV 16 L1	0.005 (± 0.012)	11	0.002 (± 0.004)	13	n.s. (0.798) ‡
HPV 18 L1	0.002 (± 0.004)	11	0.002 (± 0.004)	13	n.s. (1.000) ‡

The table shows the percentage of the antigen-specific CD4+/CD154+/ IL-4+ T cells among all CD4+ T cells. The significance of the differences between the Gardasil® and Cervarix™ group is represented by the p-values: **n.s.** = not significant; * = p < 0.05; ** = p < 0.01; *** = p < 0.001.

CD: cluster of differentiation; **IL-4:** interleukin-4; **Co.:** negative control; **HPV:** human papillomavirus; **L1:** late protein 1; **SD:** standard deviation;

‡ Statistical analysis was done using the Mann Whitney Test for independent samples.

As previously mentioned, the detection of the CD4+/CD154+/IL-4+ T cells was performed only with the negative control samples and samples stimulated with L1 peptide pools of HPV 6, 11, 16 and 18 from 11 subjects immunized with Gardasil® and 13 subjects immunized with Cervarix™. Only three women from each group showed a detectable IL-4 response in the *ex vivo* settings. Regardless of the antigen used for stimulation, the mean frequencies of the antigen-specific IL-4-producing T cells were lower than 0.01%. Statistical analysis showed no significant differences in the IL-4 response between the two groups.

3.2.4 Comparison with the results of the study from SK Pacher, 2011

The results of the study by SK Pacher, 2011, together with the *ex vivo* data from the present study are shown in Table 11 and in Figure 13.[95] SK Pacher conducted a longitudinal study with the goal to analyse and compare the ability of the two HPV vaccines to induce the HPV-specific IFNγ- and IL-2-producing T cells. The *ex vivo* assay described previously (see Chapter 2.3) was performed using the blood samples from 34 women collected at five different time points: prior to the immunization, one month after the administration of the first, second and third dose, and 6 months after the completion of the full vaccination cycle with either Gardasil® or Cervarix™. The HPV-specific T cells were barely or not at all detectable prior to the immunization. The administration of the first dose of either Gardasil® or Cervarix™ led already to an increase in the frequency of the detected HPV-specific T cells. The highest T cell frequencies were detected one month after the completion of the full vaccination cycle, with Gardasil® inducing stronger cellular immune response against the low-risk HPV types and Cervarix™ against the high-risk HPV types. Even 6 months after the completion of the full vaccination cycle, the HPV-specific T cells were detected in both groups at significantly higher frequencies than before the immunization. At this point in time no significant differences in the cellular immune response were found between the Gardasil® and Cervarix™ group.

High frequencies of CD4+/CD154+/IFNγ+ and CD4+/CD154+/IL-2+ T cells specific to HPV L1 antigens are detected up to 5.5 years after the completion of the full vaccination cycle with either Gardasil® or Cervarix™ at comparable levels to those detected 6 months after the respective vaccination. For some HPV L1 antigens even higher mean frequencies (up to 2.3-times) of the antigen-specific IFNγ- and IL-2-producing T cells are detected years after the vaccination compared to the respective frequencies detected 6 months after the vaccination.

Table 11: Mean frequency of the antigen-specific CD4+/CD154+/IFNγ+ and CD4+/CD154+/IL-2+ T cells in women prior to vaccination as well as 6 months and many years after the vaccination with either Gardasil® or Cervarix™

		HPV 6 L1	HPV 11 L1	HPV 16 L1	HPV 18 L1	HPV 31 L1	HPV 45 L1
Gardasil®							
CD4/CD154/IFNγ positive T helper cells mean frequency (± SD)	Prior to vaccination †	0.001 (± 0.002)	0.000 (± 0.000)	0.001 (± 0.002)	0.000 (± 0.000)	NR	NR
	6 months ‡	0.025 (± 0.027)	0.020 (± 0.021)	0.019 (± 0.023)	0.007 (± 0.016)	0.013 (± 0.016)	0.009 (± 0.011)
	4.5 years	0.024 (± 0.018)	0.023 (± 0.021)	0.026 (± 0.036)	0.008 (± 0.018)	0.008 (± 0.005)	0.008 (± 0.005)
Cervarix™							
CD4/CD154/IFNγ positive T helper cells mean frequency (± SD)	Prior to vaccination †	0.003 (± 0.005)	0.000 (± 0.000)	0.000 (± 0.000)	0.001 (± 0.003)	NR	NR
	6 months ‡	0.007 (± 0.006)	0.007 (± 0.006)	0.014 (± 0.011)	0.006 (± 0.008)	0.008 (± 0.006)	0.006 (± 0.009)
	4.5 years	0.016 (± 0.021)	0.012 (± 0.009)	0.020 (± 0.019)	0.011 (± 0.010)	0.008 (± 0.008)	0.010 (± 0.012)
Gardasil®							
CD4/CD154/IL-2 positive T helper cells mean frequency (± SD)	Prior to vaccination †	0.004 (± 0.005)	0.002 (± 0.004)	0.004 (± 0.005)	0.001 (± 0.003)	NR	NR
	6 months ‡	0.049 (± 0.056)	0.042 (± 0.043)	0.038 (± 0.047)	0.018 (± 0.024)	0.023 (± 0.023)	0.021 (± 0.023)
	4.5 years	0.045 (± 0.028)	0.051 (± 0.032)	0.053 (± 0.060)	0.027 (± 0.032)	0.030 (± 0.022)	0.015 (± 0.010)
Cervarix™							
CD4/CD154/IL-2 positive T helper cells mean frequency (± SD)	Prior to vaccination †	0.005 (± 0.007)	0.002 (± 0.004)	0.004 (± 0.005)	0.003 (± 0.005)	NR	NR
	6 months ‡	0.028 (± 0.026)	0.022 (± 0.012)	0.046 (± 0.036)	0.022 (± 0.015)	0.023 (± 0.016)	0.049 (± 0.085)
	4.5 years	0.045 (± 0.065)	0.033 (± 0.020)	0.058 (± 0.068)	0.037 (± 0.047)	0.016 (± 0.013)	0.036 (± 0.036)

The table shows the percentage of the antigen-specific CD4+/CD154+/INFγ+ or CD4+/CD154+/IL-2+ T cells among all CD4+ T cells.

HPV: human papillomavirus; **L1:** late protein 1; **CD:** cluster of differentiation; **IFNγ:** interferon gamma; **SD:** standard deviation; **IL-2:** interleukin-2; **NR:** not reported;

† and ‡) Data from SK Pacher, 2011.[95]

Taking into account what was said in the preceding paragraph and given the fact that the mean frequencies of the antigen-specific IFNγ- and IL-2-producing T cells 6 months after the completion of the full vaccination cycle are significantly higher than the respective frequencies among the non-vaccinated women, we can accept the conclusion that the antigen-specific IFNγ- and IL-2-producing T cells are detected even 4.5 years after the vaccination with either Gardasil® or Cervarix™ at significantly higher level than in non-vaccinated women.

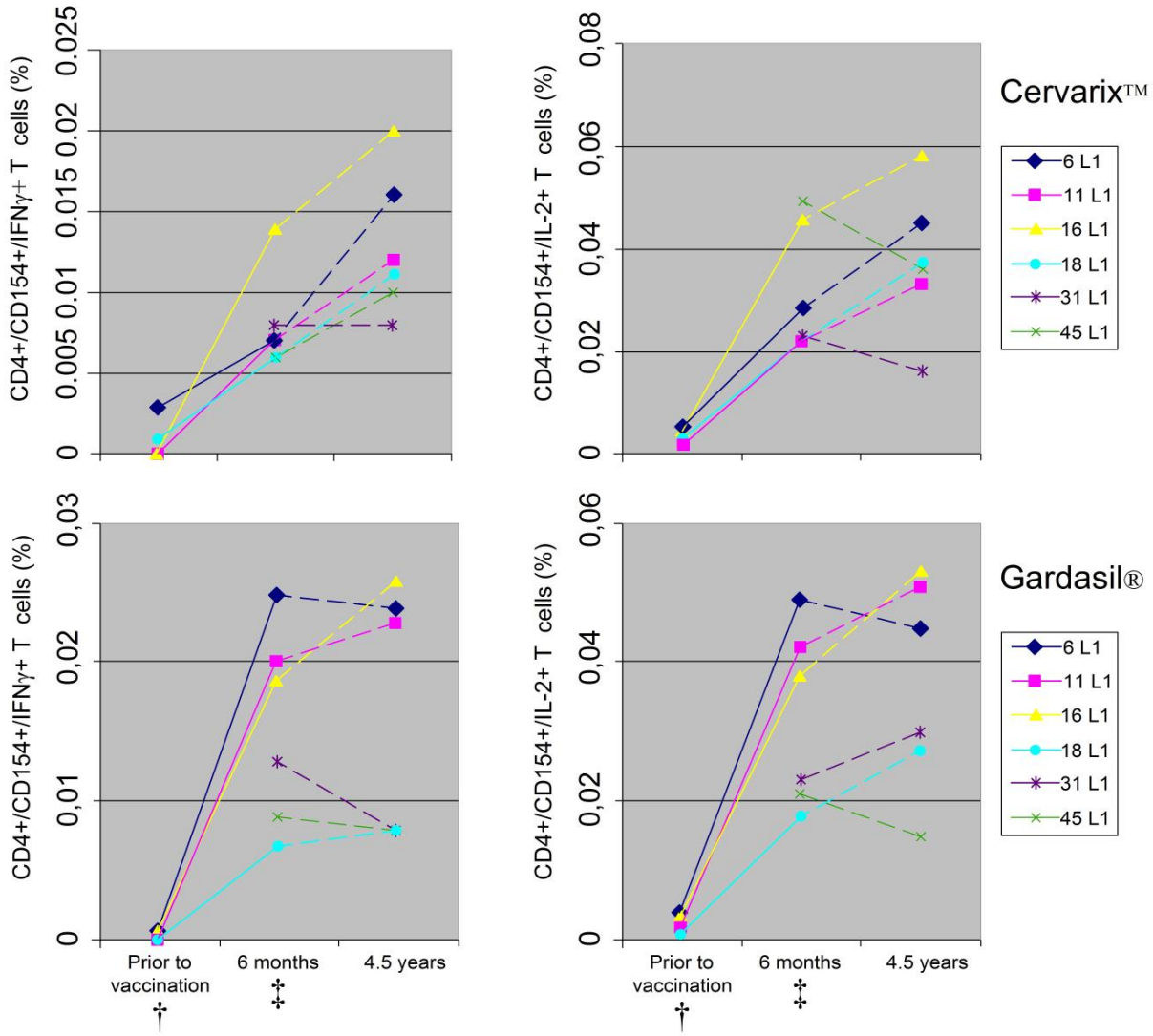

Fig. 13: **Mean frequency of the antigen-specific CD4+/CD154+/IFNγ+ and CD4+/CD154+/IL-2+ in women prior to vaccination as well as 6 months and many years after the vaccination with either Gardasil® or Cervarix™**

The figure shows the percentage of the antigen-specific CD4+/CD154+/INFγ+ or CD4+/CD154+/IL-2+ T cells among all CD4+ T cells in women prior to the vaccination, 6 months and 4.5 years after the completion of the full vaccination cycle with either Gardasil® or Cervarix™. CAVE: Data from two individual cohorts (1. cohort - prior to the vaccination and 6 months data; 2. cohort – 4.5 years data).

HPV: human papillomavirus; **L1:** late protein 1; **CD:** cluster of differentiation; **IFNγ:** interferon gamma; **IL-2:** interleukin 2;

† and ‡) Data from SK Pacher, 2011.[95]

3.3 Results of the in vitro assay

3.3.1 Definition of limits and exclusion criteria (Analysis approach)

The CD154/CD137-based *in vitro* assay was conducted in order to analyse the vaccine-specific memory CD4 and CD8 T cell response. For the samples stained with anti human CD4, CD154, IL-2 and IFNγ, the percentage of the activated CD4+/CD154+ T cells and the percentage of specifically activated CD4+/CD154+ T cells which produced IFNγ or IL-2 (double positive

cells) among all CD4+ T cells was determined. In analogy to this, for the samples stained with anti human CD8, CD137, IL-2 and IFNγ, the percentage of the activated CD8+/CD137+ T cells and the percentage of specifically activated CD8+/CD137+ T cells which produced IFNγ or IL-2 (double positive cells) among all CD8+ T cells was determined. In order to preserve the clarity of the study only the double positive cells are presented. Years after the vaccination these double positive cells represent the activated memory T cells that have been expanded in culture over limited time span of 9 to 10 days.

The double positive cells detected in the medium control samples accounted for the background stimulation and their frequencies were in almost all cases lower than the frequencies of the double positive cells detected in the antigen re-stimulation samples.

The frequency of the double positive cells needed to be higher in the samples which have been re-stimulated with the respective peptide pools (re-stimulation) compared with the samples which have encountered the antigen only on day 0 (negative control). This condition was satisfied by all subjects for the samples stained with anti human CD4, CD154, IL-2 and IFNγ. As for the samples stained with anti human CD8, CD137, IL-2 and IFNγ, higher frequencies of the double positive cells were often detected in the negative control samples compared with the respective re-stimulation samples. This showed that our *in vitro* settings did not allow clear detection of memory CD8 T cells, therefore further analysis of the CD8 data was not performed.

As previously described (see Chapter 2.4), one culture tube from each well was re-stimulated on day 9 or 10 with SEB (SEB-control) and was stained only with anti human CD4, CD154, IL-2 and IFNγ. In order to prove that the cell culture was viable, the frequency of the double positive cells in the SEB-control samples needed to be equal to or higher than the respective frequency in the antigen re-stimulation samples. In addition, the frequency of the double positive cells in the SEB-control samples needed to be at least 2.00%. This prerequisite was always satisfied.

Once all the data had been collected, statistical analysis was performed for all samples stained with anti human CD4, CD154, IL-2 and IFNγ: First, the data within each group separately was analysed (see Chapter 3.3.2). We compared the frequencies of the double positive cells in the medium control samples with the those in the antigen re-stimulation samples. In addition, we analysed whether there were differences in response between different antigen re-stimulations. Second, we compared the responses induced by the two vaccines (see Chapter 3.3.3). In order to avoid the falsification of the results caused by different levels of the background stimulation, we first subtracted the frequencies of the double positive cells in the medium control samples from

those in the antigen re-stimulation samples and then compared the resulting frequencies of the antigen-specific double positive cells between the Gardasil® and Cervarix™ group. Third, we calculated the expansion index by dividing the frequencies of the antigen-specific double positive cells from each subject by the respective frequencies measured using the *ex vivo* settings (see Chapter 3.3.4). In addition, we compared the expansion indices between the Gardasil® and the Cervarix™ group.

In order to artificially increase the number of the samples and thereby allow more accurate statistical analysis, we pooled the data into the following groups: low-risk group consisting of frequencies of the CD4+/CD154+/IFNγ+ or IL-2+ T cells specific for HPV 6 or 11; high-risk group consisting of frequencies of the CD4+/CD154+/IFNγ+ or IL-2+ T cells specific for HPV 16 or 18; cross-reactive group consisting of frequencies of the CD4+/CD154+/IFNγ+ or IL-2+ T cells specific for HPV 31 or 45; natural infection control group consisting of frequencies of the CD4+/CD154+/IFNγ+ or IL-2+ T cells specific for E6/E7 peptide from HPV 6 or 16/18. The statistical analysis described above was additionally performed using this pooled data (see Chapters 3.3.2, 3.3.3 and 3.3.4).

3.3.2 Analysis of T cell frequencies within the two groups

3.3.2.1 Gardasil®

Using the CD154-based *in vitro* assay, the HPV L1-specific CD4 T cells were detected in all 7 women immunized with Gardasil® on average 5.6 years prior to the study enrolment. Tables 12 and 13 show mean frequencies of the antigen-specific CD4+/CD154+/IFNγ+ T cells and CD4+/CD154+/IL-2+ T cells, respectively. In addition, statistical significance of the difference in response to different antigen re-stimulations is shown by the p-values.

The mean frequency of the CD4+/CD154+/IFNγ+ T cells specific for HPV 6, 11, 16 and 18 was 4.624%, 4.913%, 3.783% and 1.953%, respectively, and was 8.9-, 9.4-, 7.3- and 3.7-times higher than the mean frequency of the CD4+/CD154+/IFNγ+ T cells detected in the medium control samples (0.521%). The difference in the frequencies of the IFNγ-producing T cells between the medium control samples and the samples re-stimulated with L1 peptide pools from HPV 6, 11 or 16 was statistically significant, whereas the difference between the IFNγ response detected in the medium control samples and the IFNγ response to HPV 18 was not statistically significant. Additional analysis performed on the pooled data showed significant differences in the frequencies of the CD4+/CD154+/IFNγ+ T cells between the medium control samples and the low-risk or the high-risk group, with p-values being equal to 0.004 and 0.021, respectively. The

IFNγ response was 1.7-times stronger in the low-risk group than in the high-risk group, though this difference was statistically not significant (p = 0.150).

Table 12: Mean frequency of the CD4+/CD154+/IFNγ+ T cells in women immunized with Gardasil® detected using the CD154-based *in vitro* assay

Gardasil® IFNγ	MC	HPV 6 L1	HPV 11 L1	HPV 16 L1	HPV 18 L1	HPV 31 L1	HPV 45 L1	HPV 6 E6/E7	HPV 16/18 E6/E7
n	7	7	7	7	7	3	3	4	4
Mean freq. of the **CD4/CD154/IFNγ pos. T cells** in % (±SD)	0.521 (± 0.681)	4.624 (± 3.955)	4.913 (± 4.204)	3.783 (± 3.616)	1.953 (± 1.975)	4.147 (± 2.042)	3.910 (± 1.741)	0.513 (± 0.853)	0.220 (± 0.274)
MC		*	*	*	n.s.	*	*	n.s.	n.s.
HPV 6/11 L1	**								
HPV 16/18 L1	*	n.s.							
HPV 31/45 L1	**	n.s.		n.s.					
HPV 6/16/18 E6/E7	n.s.	***		**		**			

The table shows the percentage of the antigen-specific CD4+/CD154+/INFγ+ T cells among all CD4+ T cells detected using the CD154-based *in vitro* assay. The significance of the differences between the different antigen re-stimulations is represented by the p-values:
n.s. = not significant; ***** = p < 0.05; ****** = p < 0.01; ******* = p < 0.001.

IFNγ: interferon gamma; **MC:** medium control; **HPV:** human papillomavirus; **L1:** late protein 1; **E6/E7:** early protein 6/7; **n:** number of subjects analysed; **freq.:** frequency; **pos.:** positive; **CD:** cluster of differentiation; **SD:** standard deviation.

Cross-reactive CD4+/CD154+/IFNγ+ T cells specific for HPV 31 or 45 were detected at a mean frequency of 4.147% and 3.910%, respectively. The IFNγ response was 7.7-times stronger in the cross-reactive group than in the medium control samples, the difference being statistically significant (p = 0.001). Surprisingly, the IFNγ response to HPV 31 was 1.1-times stronger than the response to HPV 16 and the IFNγ response to HPV 45 was 2.0-times stronger than the response to HPV 18, though the difference in IFNγ response between the cross-reactive and the high-risk group was statistically not significant (p = 0.207). Of note is that when only the three women whose blood was additionally stimulated with L1 peptide pools of HPV 31 and 45 were analysed, the mean frequency of antigen-specific CD4+/CD154+/IFNγ+ T cells was the highest for HPV 16 (7.047%), followed by HPV 31 (4.147%), 45 (3.910%) and 18 (3.697%).

By contrast to the results of the *ex vivo* assay, the IL-2 response detected using the CD154-based *in vitro* assay was weaker than the corresponding IFNγ response. The mean frequency of the CD4+/CD154+/IL-2+ T cells specific for HPV 6, 11, 16 and 18 was 3.277%, 3.309%, 2.444% and 1.440%, respectively, and was 8.5-, 8.6-, 6.3- and 3.7-times higher than the mean frequency of the CD4+/CD154+/IL-2+ T cells detected in the medium control samples (0.386%). The difference in the frequencies of the IL-2-producing T cells between the medium control samples

and the samples re-stimulated with L1 peptide pools from HPV 6, 11 or 16 was statistically significant, whereas the difference between the IL-2 response detected in the medium control samples and IL-2 response to HPV 18 was not statistically significant. Additional analysis performed on the pooled data showed significant differences in the frequencies of the CD4+/CD154+/IL-2+ T cells between the medium control samples and the low-risk or the high-risk group, with p-values being equal to 0.002 and 0.046, respectively. The IL-2 response was 1.7-times stronger in the low-risk group than in the high-risk one, though this difference was statistically not significant (p = 0.096).

Table 13: Mean frequency of the CD4+/CD154+/IL-2+ T cells in women immunized with Gardasil® detected using the CD154-based *in vitro* assay

Gardasil® IL-2	MC	HPV 6 L1	HPV 11 L1	HPV 16 L1	HPV 18 L1	HPV 31 L1	HPV 45 L1	HPV 6 E6/E7	HPV 16/18 E6/E7
n	7	7	7	7	7	3	3	4	4
Mean freq. of the **CD4/CD154/IL-2 pos. T cells** in % (±SD)	0.386 (± 0.446)	3.277 (± 2.589)	3.309 (± 2.529)	2.444 (± 2.555)	1.440 (± 1.225)	3.037 (± 1.744)	3.137 (± 1.939)	0.470 (± 0.570)	0.253 (± 0.226)
MC		**	*	*	n.s.	*	*	n.s.	n.s.
HPV 6/11 L1	**								
HPV 16/18 L1	*	n.s.							
HPV 31/45 L1	**	n.s.		n.s.					
HPV 6/16/18 E6/E7	n.s.	**		*		**			

The table shows the percentage of the antigen-specific CD4+/CD154+/IL-2+ T cells among all CD4+ T cells detected using the CD154-based *in vitro* assay. The significance of the differences between the different antigen re-stimulations is represented by the p-values:
n.s. = not significant; * = p < 0.05; ** = p < 0.01; *** = p < 0.001.

IL-2: interleukin-2; **MC:** medium control; **HPV:** human papillomavirus; **L1:** late protein 1; **E6/E7:** early protein 6/7; **n:** number of subjects analysed; **freq.:** frequency; **pos.:** positive; **CD:** cluster of differentiation; **SD:** standard deviation.

Cross-reactive CD4+/CD154+/IL-2+ T cells specific for HPV 31 or 45 were detected at a mean frequency of 3.037% and 3.137%, respectively. The IL-2 response was 8.0-times stronger in the cross-reactive group than in the medium control samples, the difference being statistically significant (p = 0.002). In analogy to the IFNγ response, IL-2 response to HPV 31 was 1.2-times stronger than the response to HPV 16 and the IL-2 response to HPV 45 was 2.2-times stronger than the response to HPV 18, though the difference in IL-2 response between the cross-reactive and the high-risk group was statistically not significant (p = 0.130). Of note is that when only the three women whose blood was additionally stimulated with L1 peptide pools of HPV 31 and 45 were analysed, the mean frequency of antigen-specific CD4+/CD154+/IL-2+ T cells was the highest for HPV 16 (4.827%), followed by HPV 45 (3.137%), 31 (3.037%) and 18 (2.377%).

As far as the natural infection control group is concerned, both IFNγ and IL-2 responses were found not to differ significantly from the respective responses detected in the medium control samples.

3.3.2.2 Cervarix™

Using the CD154-base *in vitro* assay, the HPV L1-specific CD4 T cells were detected in all 7 women immunized with Cervarix™ on average 3.6 years prior to the study enrolment. Tables 14 and 15 show the mean frequencies of the antigen-specific CD4+/CD154+/IFNγ+ T cells and CD4+/CD154+/IL-2+ T cells, respectively. The statistical significance of the difference in response to various antigen re-stimulations is shown by the p-values.

Table 14: Mean frequency of the CD4+/CD154+/IFNγ+ T cells in women immunized with Cervarix™ detected using the CD154-based *in vitro* assay

Cervarix™ IFNγ	MC	HPV 6 L1	HPV 11 L1	HPV 16 L1	HPV 18 L1	HPV 31 L1	HPV 45 L1	HPV 6 E6/E7	HPV 16/18 E6/E7
N	7	7	7	7	7	3	3	4	4
Mean freq. of the **CD4/CD154/IFNγ pos. T cells** in % (±SD)	1.260 (± 1.113)	4.229 (± 3.740)	5.791 (± 5.133)	5.969 (± 4.545)	4.850 (± 4.159)	4.820 (± 1.824)	3.223 (± 0.608)	1.393 (± 2.105)	1.000 (± 0.954)
MC		n.s.	n.s.	*	n.s.	*	*	n.s.	n.s.
HPV 6/11 L1	*								
HPV 16/18 L1	*	n.s.							
HPV 31/45 L1	**	n.s.		n.s.					
HPV 6/16/18 E6/E7	n.s.	**		*		**			

The table shows the percentage of the antigen-specific CD4+/CD154+/INFγ+ T cells among all CD4+ T cells detected using the CD154-based *in vitro* assay. The significance of the differences between the different antigen re-stimulations is represented by the p-values:
n.s. = not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

IFNγ: interferon gamma; **MC:** medium control; **HPV:** human papillomavirus; **L1:** late protein 1; **E6/E7:** early protein 6/7; **n:** number of subjects analysed; **freq.:** frequency; **pos.:** positive; **CD:** cluster of differentiation; **SD:** standard deviation.

The mean frequency of the CD4+/CD154+/IFNγ+ T cells specific for HPV 6, 11, 16 and 18 was 4.229%, 5.791%, 5.969% and 4.850%, respectively, and was 3.4-, 4.6-, 4.7- and 3.8-times higher than the mean frequency of the CD4+/CD154+/IFNγ+ T cells detected in the medium control samples (1.260%). The difference in CD4+/CD154+/IFNγ+ T cells frequencies between the medium control samples and the samples re-stimulated with L1 peptide pools from HPV 16 was statistically significant, whereas the difference between the IFNγ response detected in the medium control samples and the IFNγ responses to HPV 6, 11 or 18 was not statistically significant. When pooled together, the IFNγ response detected in the low-risk or the high-risk

group was significantly higher than the response detected in the medium control samples, with p-values being equal to 0.025 and 0.012, respectively. Surprisingly, the IFNγ response was only 1.1-times stronger in the high-risk group compared with the low-risk group and this difference was statistically not significant (p = 0.709).

Cross-reactive CD4+/CD154+/IFNγ+ T cells specific for HPV 31 or 45 were detected at a mean frequency of 4.820% and 3.223%, respectively. The IFNγ response was 3.2-times stronger in the cross-reactive group than in the medium control samples, the difference being statistically significant (p = 0.003). Comparing the IFNγ responses between the high-risk and the cross-reactive group, a significant difference was not found (p = 0.207). Still it should be mentioned that the IFNγ response to HPV 16 was 1.2-times stronger than the response to HPV 31 and the IFNγ response to HPV 18 was 1.5-times stronger than the response to HPV 45.

Table 15: Mean frequency of the CD4+/CD154+/IL-2+ T cells in women immunized with Cervarix™ detected using the CD154-based *in vitro* assay

Cervarix™ IL-2	MC	HPV 6 L1	HPV 11 L1	HPV 16 L1	HPV 18 L1	HPV 31 L1	HPV 45 L1	HPV 6 E6/E7	HPV 16/18 E6/E7
n	7	7	7	7	7	3	3	4	4
Mean freq. of the **CD4/CD154/IL-2 pos. T cells** in % (±SD)	0.821 (± 0.607)	2.683 (± 1.876)	4.146 (± 3.729)	4.343 (± 3.293)	3.290 (± 2.688)	2.970 (± 0.907)	2.033 (± 0.620)	0.898 (± 1.193)	0.668 (± 0.402)
MC		**	*	*	*	*	n.s.	n.s.	n.s.
HPV 6/11 L1	**								
HPV 16/18 L1	**	n.s.							
HPV 31/45 L1	**	n.s.		n.s.					
HPV 6/16/18 E6/E7	n.s.	**		**		**			

The table shows the percentage of the antigen-specific CD4+/CD154+/IL-2+ T cells among all CD4+ T cells detected using the CD154-based *in vitro* assay. The significance of the differences between the different antigen re-stimulations is represented by the p-values:
n.s. = not significant; * = p < 0.05; ** = p < 0.01; *** = p < 0.001.

IL-2: interferon gamma; **MC:** medium control; **HPV:** human papillomavirus; **L1:** late protein 1; **E6/E7:** early protein 6/7; **n:** number of subjects analysed; **freq.:** frequency; **pos.:** positive; **CD:** cluster of differentiation; **SD:** standard deviation.

By contrast to the results of the *ex vivo* assay, the IL-2 responses detected using the CD154-based *in vitro* assay were weaker than the respective IFNγ responses. The mean frequency of the CD4+/CD154+/IL-2+ T cells specific for HPV 6, 11, 16 and 18 was 2.683%, 4.146%, 4.343% and 3.290%, respectively, and was 3.3-, 5.0-, 5.3- and 4.0-times higher than the mean frequency of the CD4+/CD154+/IL-2+ T cells detected in the medium control samples (0.821%). The differences in CD4+/CD154+/IL-2+ T cell frequencies between the medium control samples and the samples re-stimulated with L1 peptide pools from HPV 6, 11, 16 or 18 were statistically

significant. Higher levels of significance were found when the frequencies of the CD4+/CD154+/IL-2+ T cells between the medium control samples and the low-risk or the high-risk group were compared, with p-values being equal to 0.002 and 0.003, respectively. Like the IFNγ response, the IL-2 response was only 1.1-times stronger in the high-risk group than in the low-risk one, the difference being statistically not significant (p = 0.571).

Cross-reactive CD4+/CD154+/IL-2+ T cells specific for HPV 31 or 45 were detected at a mean frequency of 2.970% and 2.033%, respectively. The IL-2 response was 3.0-times stronger in the cross-reactive group than in the medium control samples, the difference being statistically significant (p = 0.005). Comparing the frequencies of the CD4+/CD154+/IL-2+ T cells specific for HPV 16 or 18 with those specific for HPV 31 or 45, the IL-2 response to HPV 16 was 1.5-times stronger than the response to HPV 31 and the IL-2 response to HPV 18 was 1.6-times stronger than the response to HPV 45, though the difference in the IL-2 response between the cross-reactive and the high-risk group was statistically not significant (p = 0.130).

As far as the natural infection control group is concerned, both IFNγ and IL-2 responses were found not to differ significantly from the respective responses detected in the medium control samples.

3.3.3 Comparison of the T cell frequencies (Gardasil® vs. Cervarix™)

3.3.3.1 Interferon-γ

As previously mentioned (see Chapter 3.3.1), in order to avoid the falsification of the results caused by different levels of the background stimulation, we subtracted the frequencies of the CD4+/CD154+/IFNγ+ T cells in the medium control samples from the those in the antigen re-stimulation samples. The resulting frequencies of antigen-specific CD4+/CD154+/IFNγ+ T cells were used to compare the IFNγ response between the Gardasil® and Cervarix™ group. For altogether 8 samples (one re-stimulation with HPV 11 L1, HPV 18 L1 and HPV 31 L1, three re-stimulations with HPV 6 E6/E7 and two re-stimulations with HPV 16/18 E6/E7), the frequency of the CD4+/CD154+/IFNγ+ T cells in the medium control samples was higher than the one in the antigen re-stimulation samples. Since cells can not be detected at a negative level, the frequency of the antigen-specific CD4+/CD154+/IFNγ+ T cells in these cases was set to 0.00%.

Table 16 shows the mean frequencies of the antigen-specific CD4+/CD154+/IFNγ+ T cells in the Gardasil® and the Cervarix™ group. The significance of the difference is shown by the p-values. In addition figure 14 visualises the differences in the frequencies of the antigen-specific

IFNγ-producing T cells between the Gardasil® and the Cervarix™ group, showing also the percentage of women in each group with detectable antigen-specific T cell response.

Table 16: Mean frequency of the CD4+/CD154+/IFNγ+ T cells in women immunized with either Gardasil® or Cervarix™ detected using the CD154-based *in vitro* assay

Antigen	GARDASIL®		CERVARIX™		Significance (p-value)
	Mean frequency of the **CD4/CD154/IFNγ positive T cells** in % (±SD)	n	Mean frequency of the **CD4/CD154/IFNγ positive T cells** in % (±SD)	n	
HPV 6 L1	4.103 (± 3.789)	7	3.057 (± 3.218)	7	n.s. (0.535)
HPV 11 L1	4.391 (± 3.939)	7	4.531 (± 4.445)	7	n.s. (0.902)
Low-risk pool	4.247 (± 3.716)	14	3.794 (± 3.806)	14	n.s. (0.734)
HPV 16 L1	3.261 (± 2.981)	7	4.709 (± 3.898)	7	n.s. (0.535)
HPV 18 L1	1.437 (± 1.352)	7	3.590 (± 3.555)	7	n.s. (0.209)
High-risk pool	2.349 (± 2.417)	14	4.149 (± 3.631)	14	n.s. (0.137)
HPV 31 L1	2.997 (± 1.634)	3	3.153 (± 2.736)	3	n.s. (0.700)
HPV 45 L1	2.760 (± 1.146)	3	1.553 (±1.711)	3	n.s. (0.400)
Cross-reactive pool	2.878 (± 1.269)	6	2.353 (± 2.221)	6	n.s. (0.818)
HPV 6 E6/E7	0.463 (± 0.847)	4	0.788 (± 1.469)	4	n.s. (0.657)
HPV16/18 E6/E7	0.170 (± 0.268)	4	0.225 (± 0.268)	4	n.s. (0.600)
Natural infection control pool	0.316 (± 0.602)	8	0.506 (± 1.023)	8	n.s. (0.983)

The table shows the mean frequency of the antigen-specific CD4+/CD154+/INFγ+ T cells among all CD4+ T cells detected using the CD154-based *in vitro* assay. The significance of the differences between the Gardasil® and the Cervarix™ group is represented by the p-values:
n.s. = not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

CD: cluster of differentiation; **IFNγ:** interferon gamma; **SD:** standard deviation; **n:** number of subjects analysed; **HPV:** human papillomavirus; **L1:** late protein 1; **E6/E7:** early protein 6/7; **low-risk pool:** HPV 6 or 11 L1; **high-risk pool:** HPV 16 or 18 L1; **cross-reactive pool:** HPV 31 or 45 L1; **natural infection control pool:** HPV 6 or 16/18 E6/E7.

The mean frequency of the CD4+/CD154+/IFNγ+ T cells specific for the low-risk HPV types 6 or 11 was 4.247% in the Gardasil® group and was 1.1-times higher than in the Cervarix™ group (3.792%). It is important to mention that although the overall frequency of the CD4+/CD154+/IFNγ+ T cells specific for either HPV 6 or 11 was higher in the Gardasil® group, the IFNγ response to only HPV 11 was slightly stronger in the Cervarix™ group (4.531% vs. 4.391%). Cervarix™ induced 1.8-times stronger IFNγ response to the high-risk HPV 16 or

18, with the mean frequency being 4.149% and 2.349% in the Cervarix™ and Garadsil® group, respectively. With a mean frequency of 2.878%, Gardasil® induced a 1.2-times stronger IFNγ response specific for HPV 31 or 45 than Cervarix™ (2.353%). The IFNγ response to E6/E7 proteins from HPV 6 or 16/18 was detected at low levels, the mean frequency of the CD4+/CD154+/IFNγ+ T cells being 0.316% and 0.506% in the Gardasil® and the Cervarix™ group, respectively. All the differences between the two groups were statistically not significant.

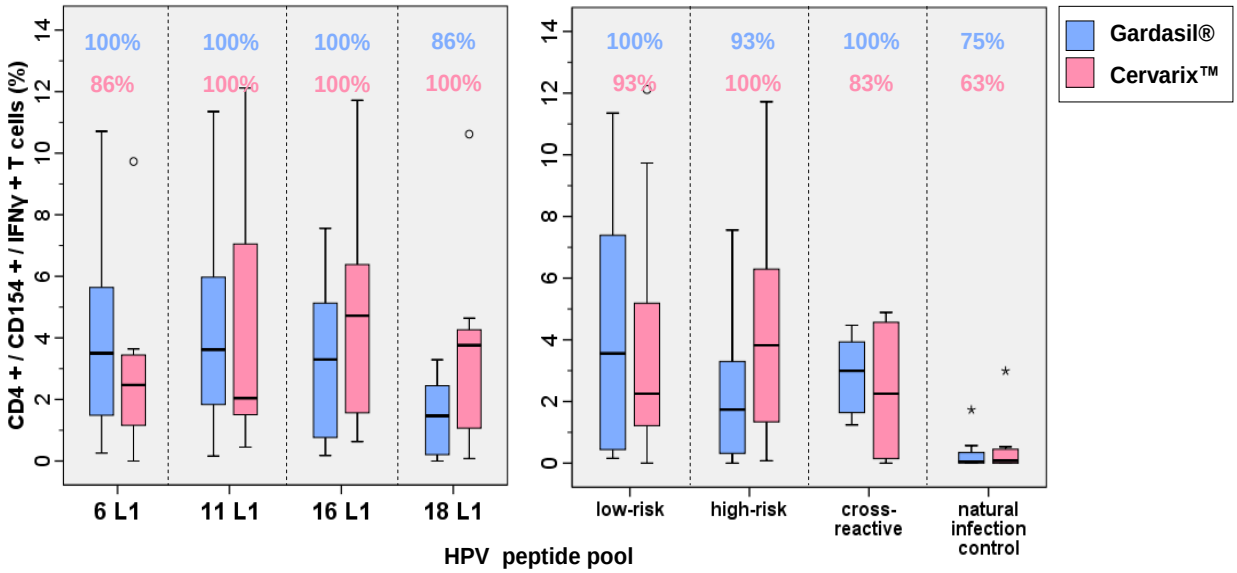

Fig. 14: Comparison of the antigen-specific CD4+/CD154+/INFγ+ T cell frequencies detected using the CD154-based *in vitro* assay between the Gardasil® and the Cervarix™ group

The figure shows the percentage of the antigen-specific CD4+/CD154+/INFγ+ T cells among all CD4+ T cells in women immunized with either Gardasil® or Cervarix™. The box represents the middle 50% of the data sample and the line in the box represents the median. The whiskers stretch both sides to a maximum of 1.5-times the interquartile range (IQR). Mild outliers up to 3-times the IQR are represented by small circles, whereas extreme outliers greater than 3-times the IQR are represented by stars.

Coloured numbers: percentage of women in each group with detectable antigen-specific T cell response;

CD: cluster of differentiation; **INFγ:** interferon gamma; **HPV:** human papillomavirus; **E6/E7:** early proteins E6/E7; **L1:** late protein 1; **low-risk pool:** HPV 6 or 11 L1; **high-risk pool:** HPV 16 or 18 L1; **cross-reactive pool:** HPV 31 or 45 L1; **natural infection control pool:** HPV 6 or 16/18 E6/E7.

3.3.3.2 Interleukin-2

In analogy to the analysis of the IFNγ response detected using the *in vitro* settings, after the frequencies of the CD4+/CD154+/IL-2+ T cells in the medium control samples were subtracted from those in the antigen re-stimulation samples, the frequencies of the antigen-specific CD4+/CD154+/IL-2+ T cells were used to compare the IL-2 responses between the Gardasil® and the Cervarix™ group. For altogether 7 samples (one re-stimulation with HPV 18 L1, two re-stimulations with HPV 6 E6/E7 and four re-stimulations with HPV 16/18 E6/E7), the frequency of the CD4+/CD154+/IL-2+ T cells in the medium control samples was higher than the one in

the antigen re-stimulation samples. In these cases, the frequency of the antigen-specific CD4+/CD154+/IL-2+ T cells was set to 0.00%.

Table 17 shows the mean frequencies of the antigen-specific CD4+/CD154+/IL-2+ T cells in the Gardasil® and the Cervarix™ group. The significance of the difference is shown by the p-values. In addition, Figure 15 visualises the differences in the frequencies of the antigen-specific IL-2-producing T cells between the two groups, showing also the percentage of women in each group with detectable antigen-specific T cell response.

Table 17: Mean frequency of the CD4+/CD154+/IL-2+ T cells in women immunized with either Gardasil® or Cervarix™ detected using the CD154-based *in vitro* assay

Antigen	GARDASIL®		CERVARIX™		Significance (p-value)
	Mean frequency of the **CD4/CD154/IL-2 positive T cells** in % (±SD)	n	Mean frequency of the **CD4/CD154/IL-2 positive T cells** in % (±SD)	n	
HPV 6 L1	2.891 (± 2.453)	7	1.861 (± 1.672)	7	n.s. (0.105)
HPV 11 L1	2.923 (± 2.352)	7	3.324 (± 3.327)	7	n.s. (0.535)
Low-risk pool	2.907 (± 2.309)	14	2.593 (± 2.641)	14	n.s. (0.533)
HPV 16 L1	2.059 (± 2.146)	7	3.521 (± 2.849)	7	n.s. (1.000)
HPV 18 L1	1.057 (± 0.834)	7	2.469 (± 2.513)	7	n.s. (0.383)
High-risk pool	1.558 (± 1.648)	14	2.995 (± 2.638)	14	n.s. (0.085)
HPV 31 L1	2.323 (± 1.276)	3	2.123 (± 1.434)	3	n.s. (0.209)
HPV 45 L1	2.423 (± 1.393)	3	1.187 (± 0.506)	3	n.s. (1.000)
Cross-reactive pool	2.373 (± 1.196)	6	1.655 (± 1.090)	6	n.s. (0.310)
HPV 6 E6/E7	0.330 (± 0.548)	4	0.295 (± 0.557)	4	n.s. (0.200)
HPV16/18 E6/E7	0.135 (± 0.193)	4	0.070 (± 0.140)	4	n.s. (0.371)
Natural infection control pool	0.233 (± 0.394)	8	0.183 (± 0.395)	8	n.s. (0.153)

The table shows the mean frequency of the antigen-specific CD4+/CD154+/IL-2+ T cells among all CD4+ T cells detected using the CD154-based *in vitro* assay. The significance of the differences between the Gardasil® and the Cervarix™ group is represented by the p-values:
n.s. = not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

CD: cluster of differentiation; **IL-2:** interleukin-2; **SD:** standard deviation; **n:** number of subjects analysed; **HPV:** human papillomavirus; **L1:** late protein 1; **E6/E7:** early protein 6/7; **low-risk pool:** HPV 6 or 11 L1; **high-risk pool:** HPV 16 or 18 L1; **cross-reactive pool:** HPV 31 or 45 L1; **natural infection control pool:** HPV 6 or 16/18 E6/E7.

The mean frequency of the CD4+/CD154+/IL-2+ T cells specific for the low-risk HPV type 6 or 11 was 2.907% in the Gardasil® group and was 1.1-times higher than in the Cervarix™ group (2.593%). Although the overall frequency of the CD4+/CD154+/IL-2+ T cells specific for either HPV 6 or 11 was higher in the Gardasil® group, the IL-2 response to only HPV 11 was 1.1-times stronger in the Cervarix™ group (3.324% vs. 2.907%). Cervarix™ induced 1.9-times stronger IL-2 response to the high-risk HPV 16 or 18, with the mean frequency being 2.995% and 1.558% in the Cervarix™ and Garadsil® group, respectively. With a mean frequency of 2.373%, Gardasil® induced 1.4-times stronger IL-2 response specific for HPV 31 or 45 than Cervarix™ (1.655%). The IL-2 response to E6/E7 proteins from HPV 6 or 16/18 was detected at low levels, the mean frequency of the CD4+/CD154+/IL-2+ T cells being 0.233% and 0.183% in the Gardasil® and Cervarix™ group, respectively. None of the differences between the two groups were statistically significant.

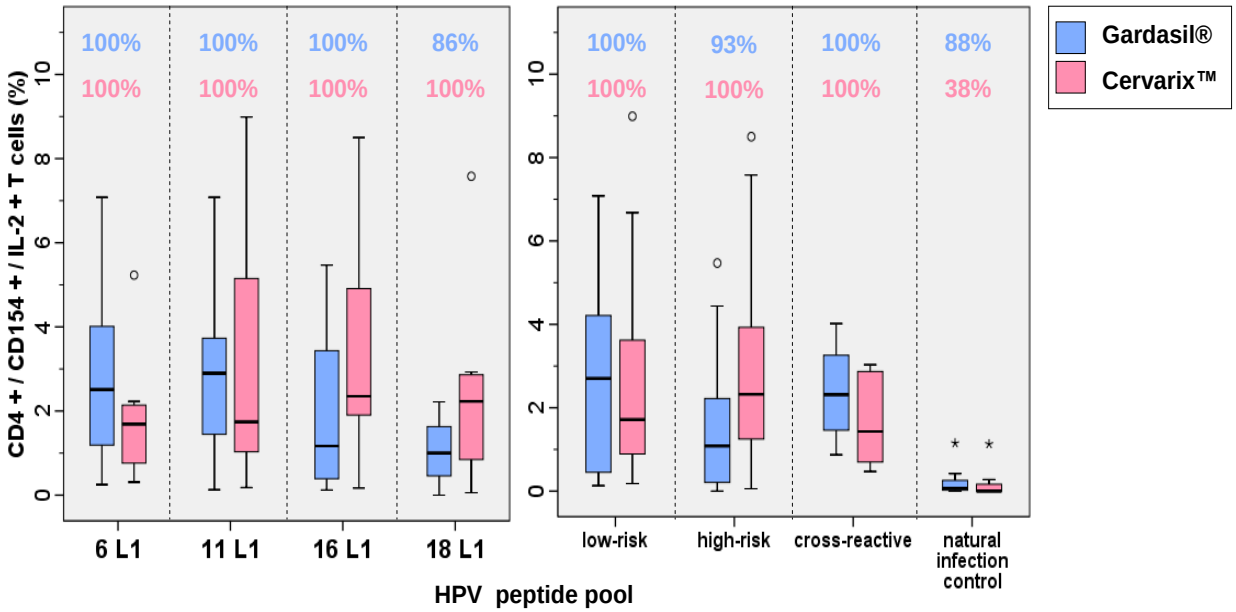

Fig. 15: Comparison of the antigen-specific CD4+/CD154+/IL-2+ T cell frequencies detected using the CD154-based *in vitro* assay between the Gardasil® and the Cervarix™ group

The figure shows the percentage of the antigen-specific CD4+/CD154+/IL-2+ T cells among all CD4+ T cells in women immunized with either Gardasil® or Cervarix™. The box represents the middle 50% of the data sample and the line in the box represents the median. The whiskers stretch both sides to a maximum of 1.5-times the interquartile range (IQR). Mild outliers up to 3-times the IQR are represented by small circles, whereas extreme outliers greater than 3-times the IQR are represented by stars.

Coloured numbers: percentage of women in each group with detectable antigen-specific T cell response;

CD: cluster of differentiation; **IL-2:** interleukin-2; **HPV:** human papillomavirus; **E6/E7:** early proteins E6/E7; **L1:** late protein 1; **low-risk pool:** HPV 6 or 11 L1; **high-risk pool:** HPV 16 or 18 L1; **cross-reactive pool:** HPV 31 or 45 L1; **natural infection control pool:** HPV 6 or 16/18 E6/E7.

3.3.4 Comparison of the expansion indices (Gardasil® vs. Cervarix™)

3.3.4.1 Interferon-γ

As explained in Chapter 3.3.1, we calculated the expansion indices by dividing the *in vitro* frequencies of the antigen-specific CD4+/CD154+/IFNγ+ T cells by the respective frequencies measured using the *ex vivo* settings. For samples in which the frequency of the CD4+/CD154+/IFNγ+ T cells was equal to 0.00% it was not possible to determine the expansion index. Most affected were the samples stimulated with E6/E7 peptide pools of HPV 6 and 16/18. Therefore, further analysis of the expansion indices of the CD4+/CD154+/IFNγ+ T cells specific for these antigens was not performed. For 6 subjects whose blood was analysed *in vitro* for the presence of the CD4+/CD154+/IFNγ+ T cells specific for HPV 31 or 45, respective *ex vivo* frequencies were not determined due to lack of material. Therefore, we were unable to determine the expansion indices of the HPV 31- or 45-specific CD4+/CD154+/IFNγ+ T cells.

Table 18 shows the mean expansion indices of the antigen-specific CD4+/CD154+/IFNγ+ T cells in the Gardasil® and the Cervarix™ group. The significance of the difference is shown by the p-values.

Table 18: Mean expansion indices of the antigen-specific CD4+/CD154+/IFNγ+ T cells in women immunized with either Gardasil® or Cervarix™

Antigen	GARDASIL®		CERVARIX™		Significance (p-value)
	Mean expansion indices of the CD4/CD154/IFNγ positive T cells (±SD)	n	**Mean expansion indices** of the CD4/CD154/IFNγ positive T cells (±SD)	n	
HPV 6 L1	280.1 (± 375.4)	7	343.8 (± 331.2)	6	n.s. (0.509)
HPV 11 L1	265.9 (± 272.3)	7	470.8 (± 440.1)	5	n.s. (0.432)
Low-risk pool	273.0 (± 315.1)	14	401.5 (± 369.7)	11	n.s. (0.217)
HPV 16 L1	142.6 (± 137.3)	7	470.9 (± 389.8)	7	* (0.038)
HPV 18 L1	113.2 (± 152.9)	3	340.8 (± 143.2)	4	n.s. (0.114)
High-risk pool	133.8 (± 134.0)	10	423.5 (± 318.8)	11	** (0.003)

The table shows the fold expansion of the antigen-specific CD4+/CD154+/IFNγ+ T cells. The significance of the differences between the Gardasil® and the Cervarix™ group is represented by the p-values: **n.s.** = not significant; * = $p < 0.05$; **= $p < 0.01$; *** = $p < 0.001$.

CD: cluster of differentiation; **IFNγ:** interferon gamma; **n:** number of subjects for which the calculation of the expansion indices was possible; **HPV:** human papillomavirus; **L1:** late protein 1; **low-risk pool:** HPV 6 or 11 L1; **high-risk pool:** HPV 16 or 18 L1.

The mean expansion index of the CD4+/CD154+/IFNγ+ T cells specific for HPV 6, 11, 16 and 18 in the Cervarix™ group was 343.8, 470.8, 470.9 and 340.8, respectively and was 1.2-, 1.8-, 3.3-, and 3.0-times higher than in the Gardasil® group. With respect to HPV 16, a significant difference in the values of the expansion indices was found between the two groups ($p = 0.038$). Analysing the HPV 16 and 18 data, the mean expansion index was 3.2-times higher in the Cervarix™ group (423.4 vs. 133.8), the difference between the two groups being highly significant ($p = 0.003$).

3.3.4.2 Interleukin-2

In analogy to the determination of the expansion indices of the antigen-specific CD4+/CD154+/IFNγ+ T cells, we calculated the expansion indices of the antigen-specific CD4+/CD154+/IL-2+ T cells. The expansion indices of the CD4+/CD154+/IL-2+ T cells specific for HPV 31 and 45 as well as for E6/E7 peptides from HPV 6 and 16/18 were not determined, reasons being the same as described in Chapter 3.3.4.1.

Table 19 shows the mean expansion indices of the antigen-specific CD4+/CD154+/IL-2+ T cells in the Gardasil® and the Cervarix™ group. The significance of the difference is shown by the p-values.

The mean expansion index of the IL-2-producing T cells specific for HPV 6 in the Gardasil® was 94.1 and was 1.3-times higher than in the Cervarix™ group (72.0), whereas the mean expansion index specific for HPV 11 was 1.8-times higher in the Cervarix™ group (110.4 vs. 62.1). In addition, Cervarix™ showed 4.6- and 2.5-times higher mean expansion index specific for HPV 16 and 18, respectively. None of the differences in the expansion indices between the two groups were statistically significant.

Table 19: Mean expansion indices of the antigen-specific CD4+/CD154+/IL-2+ T cells in women immunized with either Gardasil® or Cervarix™

Antigen	GARDASIL®		CERVARIX™		Significance (p-value)
	Mean expansion indices of the CD4/CD154/IL-2 positive T cells (±SD)	n	**Mean expansion indices** of the CD4/CD154/IL-2 positive T cells (±SD)	n	
HPV 6 L1	94.1 (± 122.3)	7	72.0 (± 85.8)	7	n.s. (0.805)
HPV 11 L1	62.1 (± 52.7)	7	110.4 (± 154.9)	7	n.s. (0.805)
Low-risk Pool	78.1 (± 92.0)	14	91.2 (± 121.9)	14	n.s. (0.874)
HPV 16 L1	39.1 (± 40.8)	7	178.4 (± 231.9)	7	n.s. (0.259)
HPV 18 L1	62.8 (± 57.4)	7	155.7 (± 269.7)	7	n.s. (0.805)
High-risk Pool	51.0 (± 49.4)	14	167.1 (± 241.9)	14	n.s. (0.223)

The table shows the fold expansion of the antigen-specific CD4+/CD154+/IL-2+ T cells. The significance of the differences between the Gardasil® and the Cervarix™ group is represented by the p-values: **n.s.** = not significant; * = $p < 0.05$; **= $p < 0.01$; *** = $p < 0.001$.

CD: cluster of differentiation; **IL-2:** interleukin-2; **n:** number of subjects for which the calculation of the expansion indices was possible; **HPV:** human papillomavirus; **L1:** late protein 1; **low-risk pool:** HPV 6 or 11 L1; **high-risk pool:** HPV 16 or 18 L1.

3.4 Serology results

3.4.1 Comparison of the mean antibody titers (Gardasil® vs. Cervarix™)

As previously mentioned, antibody titer measurements were performed using the serum samples from 14 Garadsil® and 19 Cervarix™ subjects. Following the VLP-ELISA-assay, HPV L1-specific antibodies were detected in all 33 women immunized with either Garadsil® or Cervarix™ on average 4.5 years prior to the study enrolment.

Table 20 shows the mean titers of the HPV L1-specific antibodies, together with the percentage of the subjects in each group for which the detection of the antigen-specific antibodies was possible. The significance of the difference in the HPV L1-specific antibody titers between the Garadsil® and the Cervarix™ group is shown by the p-values. In addition, the HPV L1-specific antibody titers are graphically represented in Figure 16.

Table 20: Mean antibody titers

Antigen	GARDASIL®		CERVARIX™		Significance (p-value)
	Mean antibody titer (± SD)	**%**	**Mean antibody titer** (± SD)	**%**	
HPV 6 L1	2557 (± 3414)	100	32 (± 67)	21	*** (< 0,001) ‡
HPV 11 L1	8857 (± 13822)	100	468 (± 754)	89	*** (< 0,001) ‡
HPV 16 L1	3057 (± 3400)	100	17284 (± 22475)	100	*** (< 0,001) ‡
HPV 18 L1	4443 (± 4265)	100	16568 (± 22905)	100	* (0,020) ‡
HPV 31 L1	221 (± 267)	79	279 (± 301)	74	n.s. (0,632) ‡

The table shows the mean titers of the antigen-specific antibodies from 14 Gardasil® and 19 Cervarix™ subjects. The significance of the differences between the Gardasil® and the Cervarix™ group is represented by the p-values: **n.s.** = not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

%: percentage of subjects with detectable antigen-specific antibodies; **SD:** standard deviation; **HPV:** human papillomavirus; **L1:** late protein 1;

‡ Statistical analysis was done using the Mann Whitney Test for independent samples.

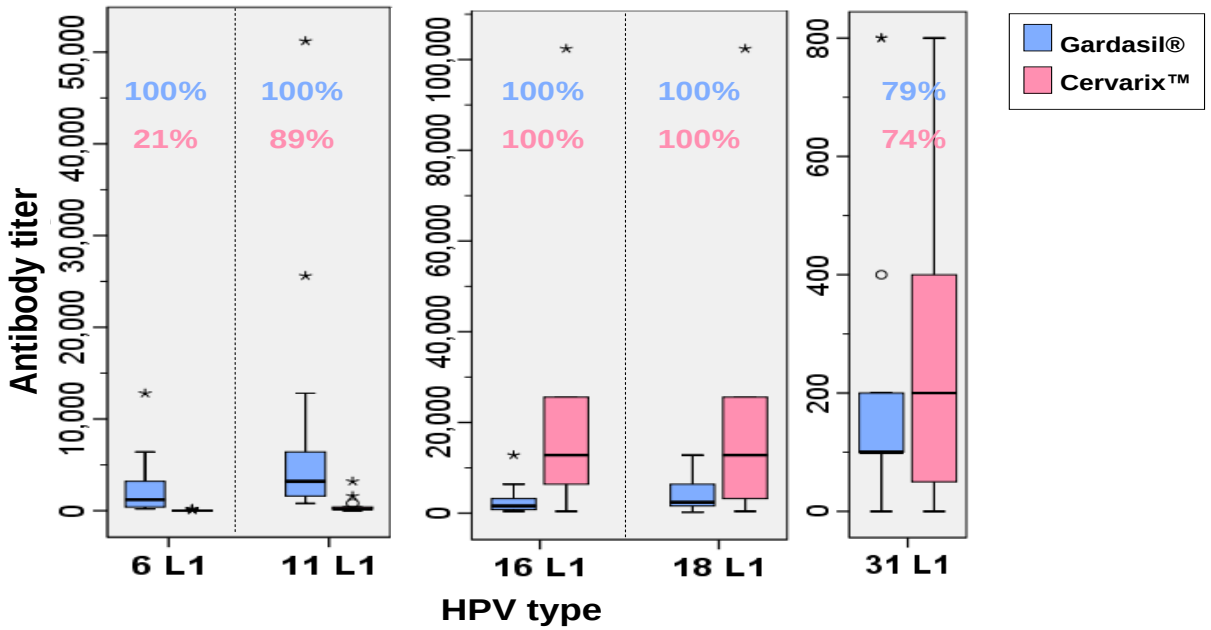

Fig. 16: Comparison of the antigen-specific antibody titers between the Gardasil® and the Cervarix™ group

The figure shows the antigen-specific antibody titers in women immunized with either Gardasil® or Cervarix™. The box represents the middle 50% of the data sample and the line in the box represents the median. The whiskers stretch both sides to a maximum of 1.5-times the interquartile range (IQR). Mild outliers up to 3-times the IQR are represented by small circles, whereas extreme outliers greater than 3-times the IQR are represented by stars.

Coloured numbers: percentage of women in each group with detectable antigen-specific humoral response;

L1: late protein 1; **HPV:** human papillomavirus.

The HPV 6 and 11 L1-specific antibodies were detected in all Gardasil® subjects, whereas only 21 and 89% of the subjects in the Cervarix™ group showed detectable serological responses to the respective peptides. The mean titers of the HPV 6 and 11 L1-specific antibodies in the Gardasil® group were 2557 and 8857, respectively. These were 80- and 19-times higher than the respective mean antibody titers detected in the Cervarix™ group (32 and 468, respectively). For both HPV 6 and 11, the difference in the antibody titers between the two groups was statistically highly significant ($p < 0.001$).

The HPV 16 and 18 L1-specific antibodies were detected in all Gardasil® as well as all Cervarix™ subjects. In contrast to serological responses to low-risk HPV types, the mean titers of the HPV 16 and 18 L1-specific antibodies in the Cervarix™ group were 5.6- and 3.7- times higher than the respective titers detected in the Gardasil® group (17284 vs. 3057 and 16568 vs. 4443, respectively). Statistically, the difference between the two groups was for HPV 16 highly significant ($p < 0.001$) and for HPV 18 significant ($p = 0.020$).

In approximately three quarters of all Gardasil® and Cervarix™ subjects, the serological responses to HPV 31 were detected. The mean titer of the HPV 31 L1-specific antibodies in the Cervarix™ group was 1.3-times higher than the respective titer in the Gardasil® group (221 vs. 279). This difference was statistically not significant ($p = 0.623$). Due to the lack of the HPV 45 L1 VLP, the detection of the HPV 45 L1-specific antibodies was not possible.

Analysing the serological responses within the Cervarix™ group, highest mean antibody titers were detected for HPV 16, followed by HPV 18. The difference between the serological response to the two high-risk HPV types was statistically not significant ($p = 0.721$). The mean antibody titers against HPV 16 and 18 were 35- to 540-times higher than those against HPV 6, 11 and 31. These differences were statistically highly significant ($p < 0.001$).

In the Gardasil® group, the highest mean antibody titers were detected for HPV 11, followed by HPV 18. The serological response to HPV 11 was 3.5-times higher than the serological response to HPV 6, the difference being statistically significant ($p = 0.018$). Surprisingly, the mean titers of the HPV 18 L1-specific antibodies in the Gardasil® group were 1.5-times higher than the mean titers of the HPV 16 L1-specific antibodies. This difference was statistically not significant ($p = 0.473$).

3.4.2 Correlation between the cellular and the humoral immune responses

The results described in the previous chapters have already shown the similarities and differences between the antigen-specific cellular and humoral immune responses induced by the two vaccines. The strongest T cell response in the Gardasil® group was detected against the HPV 16, whereas the strongest serological response was induced by the HPV 11. In the Cervarix™ group, both the strongest T cell and the strongest serological response was detected against the HPV 16.

Analysing the scatter plots (see Fig. 17) formed using the *ex vivo* frequencies of the HPV L1-specific CD4+/CD154+/IL-2+ T cells together with the HPV L1-specific antibody titers from each subject immunized with either Gardasil® or Cervarix™, a specific relationship between the cellular and the humoral immune response was not clearly visible, indicating the lack of any correlation between these two variables. Furthermore, strong outliers were found in the tails of both data sets.

In order to prove that a correlation between the cellular and the humoral immune response does in fact exist, we calculated the Spearman's rank correlation coefficient, otherwise known as the Spearman's rho for each vaccine group and each HPV L1 antigen separately. As shown on the scatter plots, in the Gardasil® group the Spearman's rho varied from -0.125 to 0.512 and the correlation between the cellular and the humoral immune response was not significant for any of the five HPV L1 antigens. In the Cervarix™ group significant correlation was found for three of the five HPV L1 antigens, with Spearman's rho being equal to -0.498 for HPV 6 L1 ($p = 0.030$), 0.592 for HPV 16 L1 ($p = 0.008$) and 0.469 for HPV 18 L1 ($p = 0.043$). The relationship between the two variables though does not seem to be linear, but rather exponential. For HPV 11 and 31 L1 in the Cervarix™ group the correlation between the cellular and the humoral immune response was not significant, with Spearman's rho being equal to -0.284 and 0.054, respectively.

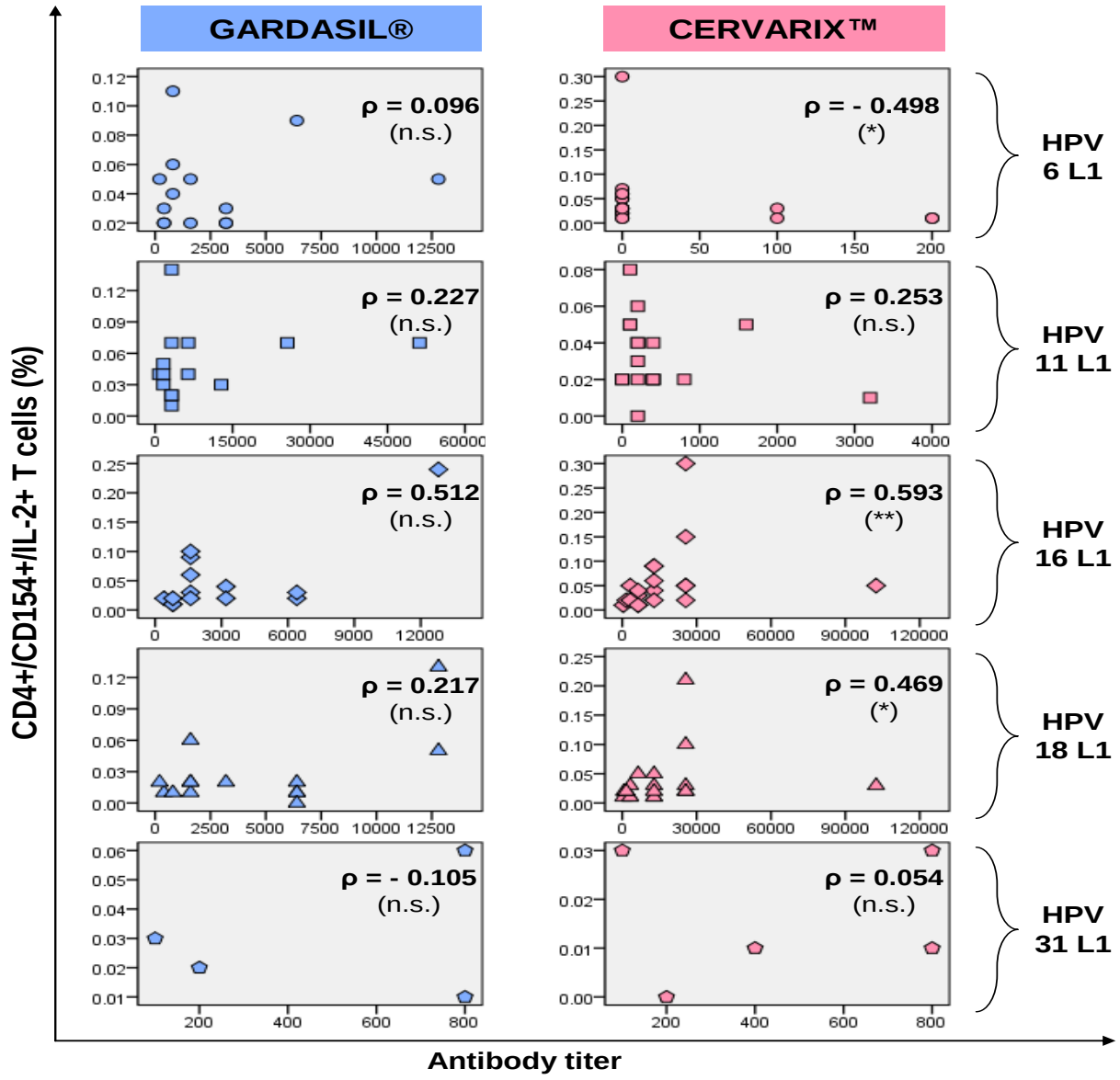

Fig. 17: Correlation between the cellular and the humoral immune responses

The figure visualizes the relationship between the *ex vivo* frequencies of the antigen-specific CD4+/CD154+/IL-2+ T cells and the antigen-specific antibody titers with the help of the scatter plots. Each point on the scatter plots represents data from one subject. The correlation between the cellular and the humoral immune response is analysed using the Spearman's rho. The statistical significance of the Spearman's rho is represented by the p-values: **n.s.** = not significant; * = $p < 0.05$; ** = $p < 0.01$.

CD: cluster of differentiation; **IL-2:** interleukin-2; **ρ:** Spearman's rank correlation coefficient (Spearman's rho); **HPV:** human papillomavirus; **L1:** late protein 1.

3.5 Detailed analysis of the data collected from the study participants with evidence of prior HPV infection

Considering the fact that a natural HPV infection in the past could alter the humoral as well as the cellular immune response induced by the two prophylactic vaccines, we divided the study

collective into two groups, the previously exposed and the naive group, in order to closer analyse the possible effects of a natural HPV infection in the past.

Analysing the questionnaires, altogether three women from the Gardasil® group reported a pathological Pap smear, one of them reporting a Pap IIID lesion, which was cured via laser treatment. None of the three women reported a history of known HPV infection. In the Cervarix™ group two women reported a pathological Pap smear on one occasion, one of them reporting a history of a positive HPV test. As the exact HPV type responsible for the development of the pathological Pap smears and lesions in any of the five women was unknown, a potential exposure to all HPV types analysed was assumed.

In order to quantify the cellular immune response to a natural HPV infection in the past, the frequencies of the CD4+/CD154+/IFNγ+ or IL-2+ T cells specific for the E6 or the E7 protein from HPV 6 and 16/18 were determined using the *ex vivo* assay and the results were presented in Chapter 3.2. For 13 women from the Gardasil® and 12 women from the Cervarix™ group, IL-2-producing T cells specific for HPV 6 E6/E7 were detected at a mean frequency of 0.019% and 0.008%, respectively. The difference between the two groups was statistically significant ($p = 0.009$). The IL-2-producing T cell responses specific for E6/E7 proteins from HPV 16 and 18 were detected in only 4 women from the Gardasil® and 5 women from the Cervarix™ group at a mean frequency of 0.003% in both groups. The IFNγ-producing T cell responses to E6/E7 proteins from both low-risk and high-risk HPV types were detected at lower levels in fewer women, the difference between the two groups being statistically not significant.

Combining the data collected using the questionnaire and the data on detected E6/E7-specific cellular immune response, altogether 13 women from the Gardasil® and 13 women from the Cervarix™ group showed signs of an exposure to low-risk HPV types in the past, whereas only 6 women from each group showed signs of prior exposure to high-risk HPV types. Figure 18 shows a graphical presentation of the difference in the antigen-specific T cell responses between the previously exposed and the HPV naive women in the Gardasil® and the Cervarix™ group separately. In the Cervarix™ group the antigen-specific T cells tend to be detected at higher frequencies among the women with no signs of prior exposure to HPV, whereas in the Gardasil® group prior exposure to HPV induces stronger antigen-specific T cell responses.

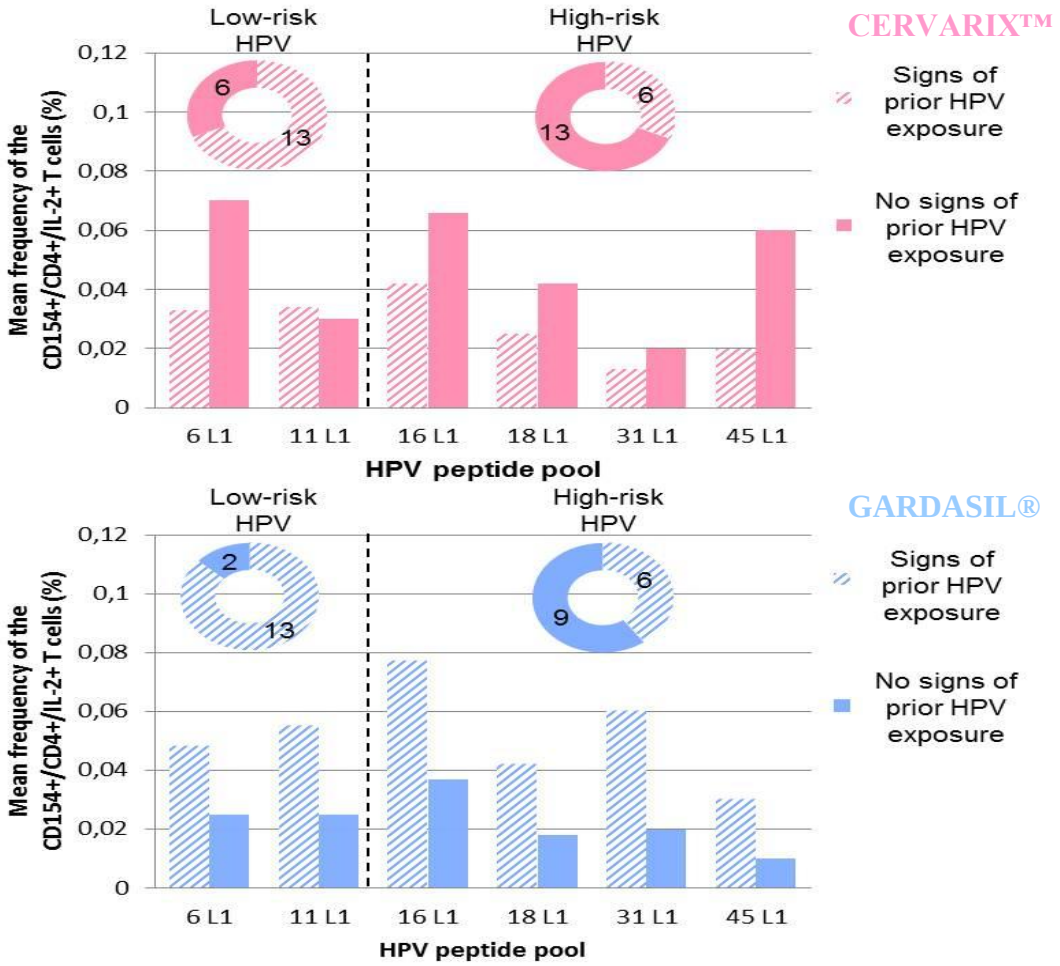

Fig. 18: Difference in the antigen-specific T cell responses between the previously exposed and HPV naive women from the Gardasil® and the Cervarix™ group

The figure visualizes the difference in the *ex vivo* frequencies of the antigen-specific CD4+/CD154+/IL-2+ T cells between the previously exposed and HPV naive women from the Gardasil® and Cervarix™ group separately. The pie charts show the number of women in each group with signs of prior exposure to low-risk or high-risk HPV types as well as the number of the women naive to the respective HPV types.

CD: cluster of differentiation; **IL-2:** interleukin-2; **ρ:** Spearman's rank correlation coefficient (Spearman's rho); **HPV:** human papillomavirus; **L1:** late protein 1.

4 DISCUSSION

4.1 Overview

The knowledge accumulated over the last three decades led to the development of the two prophylactic HPV vaccines, a quadrivalent vaccine that protects primarily against HPV 6, 11, 16 and 18 (Gardasil®) and a bivalent vaccine that protects primarily against HPV 16 and 18 (Cervarix™). Both vaccines are composed of HPV L1 proteins assembled into virus-like particles, but are produced in different cells and contain different adjuvants. Several clinical trials of both vaccines recorded very high prophylactic efficacy against the vaccine-type HPV. For additional phylogenetically related HPV-types, higher levels of cross-protection were

recorded following immunization with Cervarix™. Both vaccines were shown to be highly immunogenic, some evidence indicating that Cervarix™ induces higher levels of sustained antibody titers.

The goal of this immunologic study was to analyse and compare the long-term humoral and cellular immune responses induced by the two licensed prophylactic vaccines. The present study is methodologically based on a cross-sectional study from AK Ramseger 2009.[87] Following the *ex vivo* stimulation of whole blood from women immunized with Gardasil®, AK Ramseger managed to detect for the first time the HPV-specific T cells with the help of innovative flow-cytometry. Using the same method, a longitudinal study from SK Pacher 2011 [95] analysed and compared for the first time the HPV-specific T cell responses induced by the immunization with either Gardasil® or Cervarix™. The observation period of the latter study was limited to 6 months after the completion of the full immunization cycle (see Chapter 3.2.4).

The present cross-sectional study was conducted as a follow-up study from the longitudinal study from SK Pacher to analyse and compare the long-term memory T cell responses, allowing for the first time the detection of the HPV-specific memory T cells in women immunized with either Gardasil® or Cervarix™ on average 4.5 years prior to the study enrolment. In addition, the previously described *in vitro* assay, conducted using the blood samples from a subset of the study participants, allowed the detection of the vaccine-induced memory T cells at higher frequencies and the analysis of their expansion potentials. Furthermore, the measurements of the HPV-specific antibody titers allowed for the analysis of the correlation between the long-term humoral and cellular immune responses induced by the two prophylactic HPV vaccines.

To a great extent the two study groups were homogeneous. The difference in the age between the Gardasil® and the Cervarix™ group was found not to be statistically significant. Still it is important to mention that on average women in the Cervarix™ group were 5 years older than women in the Gardasil® group. The older age of the Cervarix™ vaccinees could have positively influenced the detected vaccine-induced immune responses, as the women of older age have had a higher risk of acquiring a natural HPV infection prior to the vaccination with Cervarix™, leading to a potentially boosted response to the subsequent vaccination. A gradual impairment of the immune response with age is plausible, negatively influencing the detected Cervarix™ induced immune responses.

The difference in the time between the completion of the full vaccination cycle and blood sampling between the two study groups was found not to be statistically significant. Including

only the 14 women whose blood was used to conduct the *in vitro* assay, the women from the Gardasil® group were immunized on average 2 years earlier, with the difference between the two groups being statistically highly significant. It is important to keep this difference in mind when analysing the results of the *in vitro* assay, as the longer time span between the immunisation with Gardasil® and blood sampling could have negatively affected the detection of the T cells. However the T cells detected years after the immunization represent the memory T cells and are expected to remain detectable at a relatively stabile plateau for years to come.

With respect to immunomodulating factors, gynaecological anamnesis and sexual behaviour, no statistically significant differences were found between the two study groups, with the exception of condom utilisation. Although condoms are not considered to provide sufficient protection against the infection with the HPV,[22,23] less frequent use of condoms in the Cervarix™ group related with higher risk of exposure to the HPV could have potentially boosted the cellular as well as the humoral vaccine-induced immune responses.

4.2 Induction of the HPV-specific memory T cells in the ex vivo settings

4.2.1 Interferon-γ and interleukin-2

Following the *ex vivo* stimulation, "double positive" T cells specific for the L1 protein from either HPV 6, 11, 16, 18, 31 or 45 were detected with the help of flow-cytometry. Years after the vaccination, these "double positive" T cells, expressing the CD154 (the general activation marker of the CD4+ T cells) as well as one of the cytokines (IFNγ, IL-2 or IL-4), represent the activated memory T helper cells.

High frequencies of the IFNγ- or IL-2-producing memory CD4 T helper cells specific to HPV L1 antigens are detected up to 5.5 years after vaccination with either Gardasil® or Cervarix™ at comparable levels to those detected 6 months after the respective vaccination (see Chapter 3.2.3).[95] Surprisingly, the T cell responses detected years after vaccination were on occasion even stronger than the responses reported 6 months after the vaccination. Over the years, vaccinated women from the present study could have been exposed to HPV more often. The higher rate of exposure could have resulted in boosted vaccine-induced memory immune responses, explaining to some extent the detection of T cells at higher frequencies. Additional analysis of the potential effects of the HPV exposure in the past was unable to provide conclusive data to support this theory (see Chapter 3.5). The prior exposure to HPV seems to

induce stronger antigen-specific T cell responses in the Gardasil® group, whereas in the Cervarix™ group the antigen-specific T cells tend to be detected at higher frequencies among the women with no signs of prior exposure to HPV. When comparing the results reported by SK Pacher with the *ex vivo* results of this study, it is important to remember that these two studies were conducted using blood samples from different individuals and that the differences in the detected T cell responses could have additionally been influenced by the interobserver variance.

In the Gardasil® group, the strongest T cells response was detected against HPV 16 and was approximately 2-times higher than the response against HPV 18. This finding largely corresponds with the published data on immunogenicity that show highest antibody titers against HPV 16. The mean antibody titer against HPV 16 five years after the first immunization with Gardasil® was reported to be almost 9-times higher than the mean antibody titer against HPV 18 and approximately 6-times higher than the mean antibody titer against HPV 6 or 11.[62] Though the difference in the reported antibody titers between HPV 16 and the low-risk HPV types is great, the difference in the detected T cell responses is minimal, the T cell response against HPV 16 being only up to 1.2-times higher than the T cell responses against the low-risk HPV types. It seems as if the memory T cells against HPV 16 are capable of inducing much stronger B cell response than the memory T cells against the low-risk HPV types. Though comparable, the data reported in the study from SK Pacher, 2011, showed the strongest T cell responses against HPV 6, followed by HPV 11, 16 and finally 18. The mean frequencies of the memory T cells against HPV 6, 11 and 16 differed minimally from each other and were 2.1- to 3.6-times higher that the mean frequency of the memory T cells against HPV 18.[95]

The strongest T cell response in the Cervarix™ group was detected against HPV 16 and was approximately 1.7-times higher than the T cell response against HPV 18. This finding corresponds with the T cell data measured 6 months after the third immunization as well as with the published data on immunogenicity, that show highest antibody titers against HPV 16.[71,95] The difference between the antibody titers against HPV 16 and 18 is less pronounced compared to the one reported in the Gardasil® immunogenicity trials. Surprisingly, the T cell response against the low-risk HPV types was detected in the Cervarix™ group at a comparable level to the T cell response against HPV 18. This finding corresponds with the T cell data measured 6 months after the third immunization.[95] Since the L1 antigens of HPV 6 and 11 are not contained in the bivalent vaccine, the question arises whether the detected “double positive” T cells are in fact specific for HPV 6 and 11. A plausible explanation for the T cell response to non-vaccine-type HPV is the cross-reactivity between the phylogenetically related HPV types.

The HPV L1 proteins contain highly conserved regions, which differ minimally from type to type. Considering the fact that the L1 protein of all HPV types serves the same function, namely the formation of the icosahedral capsid structure, it is not surprising that most HPV L1 proteins are of similar size and share significant homology.[96,97] According to the databank of the European Molecular Biology Network (EMBnet), there is a 61 - 88 % homology in the L1 amino acid sequence between the high-risk HPV 16 or 18 and the low-risk HPV 6 or 11 (see Table 21). In contrast to the high homology of the conserved linear epitopes, the conformational epitopes are type-specific and are considered to be the main target of the vaccine-induced neutralizing antibodies.[84] Both conserved linear and highly variable conformational epitopes play a role in the induction of humoral and cellular immune responses, though the shared epitopes found on the surface of phylogenetically related HPV types are likely to be less immunogenic.[83]

Table 21: Homology of the L1 amino acid sequence between various HPV types (in %)

HPV	6	11	16	18	31	45
6	100	92	86	63	68	64
11		100	68	61	69	63
16			100	65	83	64
18				100	65	88
31					100	64
45						100

In order to understand the potential mechanism that leads to the development of cross-reactivity on the cellular basis, it is important to closer analyse the function of the MHC II. Following intramuscular injection, vaccine VLPs are rapidly phagocytosed by circulating APCs, processed and, after migration to the local lymph nodes, both conserved and variable epitopes are presented on the MHC II for recognition by naive CD4+ T cells. As a result of the complex interaction with APCs, the naive CD4+ T cells differentiate into specific T helper cells (see Chapter 1.4.2). The 2nd and the 3rd vaccine doses subsequently induce a clonal expansion of these specific T helper cells.[95] The MHC II is genetically highly polymorphic and the specificity of the peptide binding to the MHC II is relatively poor, i.e. slight variations from the ideal amino acid sequence of the binding peptide are tolerated. Upon exposure to non-vaccine type HPV (natural infection or, in our case, *ex vivo* stimulation with various HPV L1 peptide pools), this lack of "MHC II binding accuracy" together with high homology of the conserved epitopes of the phylogenetically related HPV can result in efficient recognition of the non-vaccine type HPV epitopes by the vaccine-induced specific T helper cells. In other words, exposure to non-vaccine type HPV can result in a clonal expansion of the T cells primarily induced against the conserved

epitopes of the vaccine-type HPV. In such manner, the immunization with Cervarix™ can induce cellular immune responses that are potentially able to react with low-risk HPV 6 and 11.

In analogy to the cross-reactivity between the high-risk and the low-risk HPV types in the Cervarix™ group, the high T cell response to HPV 6 and 11 in the Gardasil® group could be explained in the same manner. The L1 protein of HPV 6 is 92% homologous to the L1 protein of HPV 11. Thus, the T helper cells primarily induced against the L1 protein of HPV 6 could have recognised the amino acid sequence of the L1 protein of HPV 11, inducing their clonal expansion. The same is true for HPV 11-specific T helper cells that show cross-reactivity against HPV 6.

The cross-reactivity is observed not only between the high-risk and the low-risk HPV types, but also between the phylogenetically related high-risk HPV types in both the Gardasil® and the Cervarix™ group. It has been shown that the L1 protein of HPV 16 is 83% homologous to the L1 protein of HPV 31 [82,98] and the L1 protein of HPV 18 is 88% homologous to the L1 protein of HPV 45.[83] Years after the immunization with either Gardasil® or Cervarix™, the T cell responses against HPV 31 were approximately 2- to 3- times lower than the T cell responses against the vaccine-type HPV 16, whereas the T cell responses to HPV 45 were only slightly lower than the T cell responses to the vaccine-type HPV 18. These results are comparable to those reported 6 months after the completion of the full vaccination cycle with the respective vaccine, with the exception of the T cell response to HPV 45 in the Cervarix™ group, which was 2-times higher that the T cell response to HPV 18. Additive cross-reactivity was reported as a possible explanation for this finding, though it was not seen as a sufficient explanation for the high frequencies of the HPV 45-specific T helper cells.[95] For further clarification of the biological importance of the cross-reactive T cells, analyses of the cellular immune responses to single epitopes are needed.

Comparing the frequencies of the antigen-specific IFNγ- or IL-2-producing T helper cells detected using the *ex vivo* settings, no statistically significant differences were found between the Gardasil® and the Cervarix™ group. The IFNγ- or IL-2-producing T helper cells against the high-risk HPV types (HPV 16 and 18) were detected at 1.1- to 1.4-fold higher mean frequencies in the Cervarix™ group, with the exception of the IFNγ-producing T helper cells against HPV 16 that were detected at 1.3-fold higher mean frequency in the Gardasil® group. The corresponding trend with detection of higher T cell frequencies against the high-risk HPV in the Cervarix™ group has been reported by SK Pacher.[95] The study from Einstein et. al. comparing the

serological immune responses induced by the two vaccines showed that Cervarix™ induces 2.3- to 4.8-fold and 6.8- to 9.1-fold higher GMTs of serum neutralizing antibodies against HPV 16 and 18, respectively, than Gardasil®, the difference being statistically highly significant. In addition, the HPV 16- and 18-specific memory B cells were detected at higher frequencies after the vaccination with Cervarix™ compared with Gardasil®.[65] The adjuvant system AS04, comprised of aluminium hydroxide and monophosphoryl lipid A (MPL), is likely to be the key of the enhanced immune responses induced by the vaccination with Cervarix™. Recent work has shown that AS04 adjuvanted vaccines induce up to 8.5-fold higher mean antibody titers and up to 5.2-fold stronger memory B cell responses than vaccines adjuvanted only with aluminium salts.[99] In addition data has been published demonstrating that MLP in the vaccine can enhance the quality of the antigen-specific T cell response.[100]

The T cell responses to the low-risk HPV types (HPV 6 and 11) were 1.5- to 1.9-fold stronger in the Gardasil® group, with the exception of the IL-2 positive T cell response to HPV 6 that was detected at equal levels in both groups. The corresponding trend with detection of stronger T cell responses against the low-risk HPV in the Gardasil® group has been reported by SK Pacher.[95] Taking into account that the VLPs from the low-risk HPV types are not contained in Cervarix™, the trend indicating the superiority of the T cell response against the low-risk HPV types in the Gardasil® group is understandable. In addition, the analysis of the IL-2-producing T cell response to E6/E7 peptide from HPV 6 showed a significantly higher rate of prior exposure to low-risk HPV among the study participants immunized with Gardasil®, potentially resulting in boosted vaccine-induced immune response against the HPV 6. The results shown in Chapter 3.5 support this theory, since higher levels of antigen-specific T helper cells are detected among women with signs of prior exposure to HPV in the Gardasil® group.

Comparing the cellular immune response to homologous high-risk HPV types, up to 2.4-fold stronger T helper cell response to HPV 45 was detected in the Cervarix™ group, whereas Gardasil® induced up to 1.9-fold stronger T helper cell response to HPV 31. With respect to HPV 45, the detected trends have previously been reported, whereas data from other studies show that Cervarix™ induces stronger humoral as well as cellular immune responses against HPV 31. Approximately 2.7-fold higher T helper cell frequencies against HPV 31 and 45 as well as 1.5- and 2.7-fold higher antibody titers against HPV 31 and 45, respectively, were reported following immunization with Cervarix™ compared with Gardasil®. In addition, higher clinical efficacy against the infection with, and lesions caused by, the homologous high-risk HPV types has been reported for Cervarix™. A possible explanation for the relatively strong T cell response

to HPV 31 in the Gardasil® group is the cross-reactivity with HPV 16, since the vaccination with Gardasil® induced the strongest cellular immune response to this vaccine-type HPV.

As previously mentioned, no statistically significant differences in the frequencies of the antigen-specific IFNγ- or IL-2-producing T helper cells were found between the Gardasil® and the Cervarix™ group. A plausible explanation for the lack of significance is the small number of subjects and great standard deviation in our study. It would be advisable to reassess the presented results on the T cell responses by conducting a study with a larger number of subjects in order to accept or reject the statistical trends observed.

Although the importance of the differences in magnitude of the immune response between Gardasil® and Cervarix™ is unknown, they may represent the determinants of duration of the protection against various HPV types. Long-term studies evaluating the duration of prophylactic efficacy after vaccination are needed for both vaccines.

4.2.2 Interleukin-4

Years after the vaccination, IL-4-producing memory CD4 T helper cells specific to vaccine-type HPV L1 antigens were detected only in three women from the Cervarix™ and three women from the Gardasil® group. Regardless of the antigen used for stimulation, the mean frequencies of the specific IL-4-producing T cells were lower than 0.01% in both groups. The IL-4-producing T cell responses 6 months after the completion of the full vaccination cycle with one of the two vaccines have been reported to be much lower than the IL-2- and IFNγ-producing T cell responses.[95]

There are two possible explanations: 1) The sensitivity of the CD154-based *ex vivo* assay is inadequate for the detection of this interleukin; 2) The Th2 cell response induced by the two prophylactic vaccines is less pronounced than the Th1 cell response, correlating with the low detection of IL-4-producing Th2 cells and a reliable detection of IL-2- and IFNγ-producing Th1 cells. In order to better understand the vaccine-induced Th2 cell responses, establishment of a more sensitive assay, including the additional detection of other Th2-specific markers, is needed.

4.3 Induction of the HPV-specific memory T cells in the in vitro settings

4.3.1 CD4+ T cells

Years after the vaccination, the "double positive" CD4 and CD8 T cells, expressing either CD154 or CD137 as well as one of the cytokines (IFNγ or IL-2), represent the activated memory T helper cells and the activated memory cytotoxic T lymphocytes (CTL), respectively.

Using the *in vitro* assay, we succeeded in expanding and detecting the CD4+/CD154+/IFNγ+ or IL-2+ T cells specific for the L1 protein from either HPV 6, 11, 16, 18, 31 or 45 at much higher frequencies compared to those detected using the *ex vivo* assay. In contrast to the result of the *ex vivo* assay, the IL-2 responses detected using the *in vitro* assay were weaker than the respective IFNγ responses. Pinto et al. compared the cytokine responses detected in whole blood and isolated PBMC cultures 7 months after the immunization with monovalent HPV 16 L1 vaccine. The relative increases in the detection of IL-2 following the vaccination were much higher when whole blood samples were used, whereas the relative increases in the detection of IFNγ in whole blood from vaccine recipients were approximately equal to those seen in isolated PBMC.[101] The difference in cytokine responses obtained with whole blood or PBMC might be explained by the different composition of leukocyte subsets, whole blood containing much higher levels of granulocytes and lower levels of lymphocytes compared to PBMC. In addition, activation of leukocytes in whole blood and PBMC may follow different kinetics.[102]

In the Gardasil® group the mean frequency of the antigen-specific CD4+/CD154+/IFNγ+ or IL-2+ T cells varied from 1.440 to 4.913% and was 3.7- to 9.4-fold higher than the respective mean frequency detected in the medium control samples. The differences in the detected antigen-specific T cell frequencies between the re-stimulated samples and the medium control samples were statistically significant for all L1 peptide pools analysed, with the exception of the HPV 18 L1. The lack of significance could be explained by the variability and noise in the media background responses combined with generally low cellular immune responses to HPV 18, possibly correlated with the waning seropositivity against this HPV type over time. The strongest antigen-specific T cell response was detected against the HPV 11, followed by HPV 6, 16 and 18. Interestingly, the antigen-specific T cells against the homologous high-risk HPV types were detected at higher mean frequencies than those against the HPV 18. The weaker expansion of the HPV 18-specific T cells could not be explained by the lower starting frequency, since the IFNγ responses against HPV 18 and 45 detected using the *ex vivo* assay were comparable and the

IL-2-producing T cells against HPV 18 were detected at 1.8-fold higher mean frequencies than those against HPV 45.

In the Cervarix™ group the mean frequency of the antigen-specific CD4+/CD154+/IFNγ+ or IL-2+ varied from 2.033 to 5.969% and was 2.5- to 5.3-fold higher than the respective mean frequency detected in the medium control samples. The differences in the detected antigen-specific IL-2-producing T cell frequencies between the re-stimulated samples and the medium control samples were statistically significant for all L1 peptide pools analysed, whereas the differences in the IFNγ-producing T cell response were statistically significant for only HPV 16, 31 and 45. The lack of significance for remaining HPV types could be due to the high background T cell responses detected in the medium control sample combined with low number of subjects and great standard deviation. The strongest antigen-specific T cell response was detected against the HPV 16, followed by HPV 11, 18 and 6. Interestingly, though the VLPs from the low-risk HPV types are not contained in the vaccine, the detected *in vitro* T cell responses against HPV 11 were stronger than those against HPV 18. A possible explanation is the alteration of the vaccine-induced immune responses caused by prior exposure to one of the two HPV types. Furthermore, additive cross-reactivity with homologous HPV types could result in stronger induction of the cellular immune responses against HPV 11, since HPV 11 shares on average 74.5% homology in the L1 amino acid sequence with other 5 HPV types analysed in this study, whereas HPV 18 shares on average only 68% sequence homology. With respect to homologous high-risk HPV types, the T cell responses against HPV 31 and HPV 45 were up to 1.6-fold lower than those against HPV 16 and 18, respectively. Though the difference in the detected cellular immune response is modest, the reported HPV 31 and 45 neutralizing titers 6 months after the completion of the full vaccination cycle with Cervarix™ are approximately 100-fold lower than those observed for HPV 16 and 18.[103] Despite these comparably low antibody titers, prophylactic efficacy of up to 92% against HPV 31 and 100% against HPV 45 has been reported.[73]

Comparing the T cell responses detected using the *in vitro* assay, no significant differences were found between the two groups. Gardasil® induced higher mean frequencies of the antigen-specific T cells against HPV 6 and 45, whereas the T cell responses against HPV 11, 16 and 18 were stronger among Cervarix™ vaccinees. Possible explanation for detection of stronger T cell response against HPV 11 in the Cervarix™ group is more common exposure to HPV 11 among the Cervarix™ vaccinees. Furthermore the adjuvant system AS04 contained in Cervarix™ could

be responsible for stronger expansion *in vitro*, since the HPV 11-specific T cells were detected *ex vivo* at higher mean frequencies in the Gardasil® group.

Expansion indices in the Gardasil® group (the frequency of the antigen-specific T cells detected using the *in vitro* assay divided by the respective frequency detected using the *ex vivo* assay) varied from 39 to 280 and were 1.8- to 4.3-fold higher for IFNγ compared with IL-2. In addition, antigen-specific T cells against low-risk HPV types expanded better than those against the high-risk HPV types. Expansion indices in the Cervarix™ group varied from 72 to 471 and were 2.2- to 4.8-fold higher for IFNγ compared with IL-2. In comparison to Gardasil®, antigen-specific T cells against high-risk HPV types expanded better than those against the low-risk HPV types. Though the differences between the two groups were statistically not significant, expansion indices in the Cervarix™ group were 1.2- to 4.8-fold higher than in the Gardasil® group. The adjuvant system AS04 is likely to be the key of the enhanced immune responses induced by the vaccination with Cervarix™.

The T cells specific for the E6/E7 proteins from HPV 6 and 16/18 were detected *in vitro* at frequencies comparable to those detected in the medium control samples, indicating either the lack of prior exposure of the investigated individuals to these HPV types or the inability of the E6/E7-specific T cells to expand in our *in vitro* settings.

Extrapolating the results from our *in vitro* assay, it is reasonable to assume that the *in vivo* exposure to vaccine type HPV as well as to homologous high-risk HPV 31 and 45 results in boosting of the vaccine-induced memory T cell responses. These responses are important for both the induction and maintenance of the humoral responses and might play a significant role in the outcome of the vaccination and the duration of protection against infection.

4.3.2 CD8+ T cells

The CD8+ T cell response is regarded to be important in providing protection against HPV. The CD8+ T cells have been found in CIN lesions as well as in cervical carcinoma. Therefore, we made an attempt to analyse the potential vaccine-induced CTL response by measuring the *in vitro* frequencies of the CD8+/CD137+/ IFNγ+ or IL-2+ T cells. Our *in vitro* settings did not allow sensitive detection of memory CD8+ T cells.

The possible explanations are: 1) The sensitivity of the CD137-based *in vitro* assay is inadequate for the detection of the CD8+ T cells; 2) The CD8+ T cells possibly require more rounds of

restimulation in order to expand *in vitro*; 3) The CD8+ T cell response induced by the two prophylactic vaccines is less pronounced than the CD4+ T cell response, correlating with the low detection of CD8+ T cells and a reliable detection of CD4+ T cells. In order to better understand the vaccine-induced CD8+ T cell responses, establishment of a more sensitive assay, including the additional detection of other CTL-specific markers, is needed.

4.4 Humoral immune response and its correlation with the cellular immune response

Following the VLP-ELISA-assay, antibodies against the respective vaccine type HPV L1 proteins were detected in all 33 women immunized with either Gardasil® or Cervarix™ on average 4.5 years prior to the study enrolment.

The strongest humoral immune response in the Gardasil® group was detected against HPV 11, followed by HPV 18, 16 and 6. In contrast to our results, five years after the immunization with Gardasil® the highest GMT in the per protocol population was reported against HPV 16, being approximately 6-fold higher than the GMT against the vaccine type low-risk HPV and approximately 9-fold higher than the GMT against HPV 18.[62] Since the standard deviation of the antibody titers against HPV 11 in our study has a high value, the humoral immune response of some women who have been previously exposed to HPV 11 could have been boosted by the immunization with Gardasil®, resulting in detection of very high antibody titers that distort the mean value. It is worth noting that in a study from FUTURE-group the placebo recipients who were seropositive and PCR-negative for the respective HPV type at baseline five years later showed the highest GMT against HPV 11, being 4.6- to 9.4-fold higher than the GMT against HPV 6, 16 and 18.[62]

In the Cervarix™ group the mean antibody titer against HPV 16 was slightly higher than the one against HPV 18. Corresponding results have been reported up to 6.4 years after vaccination with Cervarix™, the antibody concentrations against HPV 16 and 18 being at least 13-fold and at least 12-fold higher than the respective antibody concentrations detected after clearance of a natural infection.[61]

Comparing the long-term humoral immune response to high-risk HPV types for the first time, 3.7- to 5.7 fold higher mean antibody titers were detected in the Cervarix™ than in the Gardasil® group, the difference between the two groups being statistically significant. A recent study including 1106 women showed that GMTs of antibodies against HPV 16 and 18 measured

one month after the completion of the full vaccination cycle were 2- to 9-fold higher for Cervarix™ compared to Gardasil®. Whether these differences in the humoral immune response to Gardasil® and Cervarix™ will correlate with duration of protection afforded by the respective HPV vaccination remains to be determined.

Interestingly, 21 and 89 % of the Cervarix™ vaccinees showed detectable humoral immune response against HPV 6 and 11, respectively. Still, the mean antibody titers against HPV 6 and 11 were 19- and 80-fold higher in the Gardasil® group, the difference between the two groups being statistically highly significant. Since the antibodies against HPV 11 were detected in 4-times as many Cervarix™ vaccinees than the antibodies against HPV 6, although the natural infection with HPV 6 is more common than the natural infection with HPV 11, it is reasonable to assume that the humoral immune response detected in the Cervarix™ vaccinees is vaccine-induced. In order to determine whether the humoral and the cellular immune response to HPV 6 and 11 detected years after the immunization with Cervarix™ will be sufficient in providing protection against infection with, and lesions caused by, these low-risk HPV types long term efficacy studies are needed.

As far as the humoral response to homologous HPV types is concerned, in approximately 75% of both Gardasil® and Cervarix™ vaccinees antibodies against HPV 31 were detected. The mean antibody titer against HPV 31 was just 1.3-fold higher in the Cervarix™ group, the difference between the two groups being statistically not significant. To date, higher prophylactic efficacy against infection with and lesions caused by HPV 31 has been reported for Cervarix™. Compared to the mean antibody titers against HPV 16, the mean antibody titers against HPV 31 in the Gardasil® and the Cervarix™ groups were 14- and 62-fold lower, respectively. Corresponding data has been reported 6 months after the completion of the full vaccination cycle with Cervarix™, neutralizing antibody titers against phylogenetically related HPV types being typically 100-fold lower than those observed against the vaccine-type HPV.

As previously mentioned, the strongest T cell response in the Gardasil® group was detected against the HPV 16, whereas the strongest serological response was induced by the HPV 11. No correlation between the antigen-specific frequencies of the Th1 cells and the antibody titers was found among Gardasil® vaccinees, suggesting the heterogeneity of the immune response to vaccination between the individuals. In the Cervarix™ group both the strongest Th1 cell and the strongest serological response was detected against the HPV 16. A significant correlation was found for three of the five HPV L1 antigens, with Spearman's rho being equal to -0.498 for HPV

6 L1, 0.592 for HPV 16 L1 and 0.469 for HPV 18 L1. The relationship between the two variables though does not seem to be linear, but rather exponential. Since the Th2 cells are mainly responsible for the activation of the B cells and their further differentiation into antibody secreting plasma cells, the correlation between the antigen-specific Th2 cell and antibody response is expected to be much more pronounced and should be tested by adequate methods.

4.5 Limitations of the study

To assess the long-term immune response to the two vaccines, we conducted a non-blinded, non-randomised, cross-sectional study, including 34 women immunized with one of the two HPV vaccines on average 4.5 years prior to the study enrolment.

Due to the small number of staff members, an observer-blinded study was not possible. Furthermore, the randomisation of the study participants was not possible for two reasons: 1) The ethics committee approved a purely observational study, allowing us to only recruit women who got vaccinated on their own initiative; 2) The pure interest in the long-term immune response forced us to recruit women who had been immunized with either Gardasil® or Cervarix™ 4 to 6 years prior to the study enrolment. Despite the lack of randomisation, the two study groups showed no significant differences with respect to age, time between the completion of the full vaccination cycle and blood sampling, as well as immunological and gynaecological status.

The information gathered using the questionnaire, incl. the number of sexual partners or the evidence of prior infection with HPV, did not lead to the exclusion of the study participants. The two study groups therefore represent the general population, but not the target group of the HPV vaccination programme (HPV naive girls or women).

In the immunogenicity and efficacy trials of the two HPV vaccines, the primary analysis was conducted in the according-to-protocol populations which consisted of subjects who were PCR and seronegative to HPV at study enrolment (target group of the HPV vaccination programme). The cervical smears and serological tests with the goal to identify the HPV naive subjects were not performed in our study. A possible prior exposure to HPV was determined by analysis of the questionnaire and detection of the T cells against the early proteins E6 and E7 from HPV 6, 16 and 18. Due to the lack of E6/E7 peptide pools from HPV 11, 31 and 45, a prior infection with these HPV types was solely determined by analysis of the questionnaire. Potential boosting of already existent cellular immunity could have led to the detection of the antigen-specific T cells

at higher frequencies in study participants who have been exposed to one or more HPV types in the past. At this point it is important to mention that approximately 70% and 85% of the women in the Cervarix™ and the Gardasil® group, respectively, showed evidence of prior exposure to at least one HPV type. No significant differences were found between the two groups with respect to the T cell frequencies against the E6 and E7 proteins, with the exception of the HPV 6 E6/E7-specific CD4+/CD154+/IL-2+ T cells that were detected at 2.4-fold higher mean frequency in the Gardasil® group. Analysing the data collected using the questionnaire, the two study groups showed high homogeneity, the only significant difference being found with respect to the condom use.

In order to activate the vaccine-specific memory T cell responses, HPV peptide pools consisting of multiple 30mer peptides, which share a sequence overlap between each other and together represent the L1 wild type protein of the respective HPV type, were used in the *ex vivo* and *in vitro* assays. In contrast, both HPV vaccines consist of VLPs. A current study comparing the use of peptide pools with VLPs has shown comparably good induction of T cell responses, with slightly higher T cell frequencies being detected when VLPs are used (Tina Kube, personal communication). The use of HPV peptide pools for the activation of the vaccine-specific memory T cell responses therefore appears to be a legitimate alternative to vaccine VLPs.

With the goal to expand and thereby detect the vaccine-specific memory CD4+ T cells we conducted the previously described *in vitro* assay. Though the sensitivity in the detection of the T cells of this assay was higher compared with the *ex vivo* assay, the high levels of background stimulation still remain a problem when analysing the frequencies of the vaccine-induced memory T cells.

Despite the limitations, this study provided us with reliable data on the long-term cellular as well as humoral immune responses induced by the two prophylactic HPV vaccines. In order to better understand the biological relevance of the T cells specific for the vaccine-type HPV and the homologous HPV types, larger studies with more sensitive assays are needed. These should include only the HPV naive girls or women, in order to avoid the falsification of the results caused by prior HPV exposure. Furthermore, the correlation between the vaccine-induced cellular and humoral immune responses should be analysed, with a special focus on the memory Th2 cells. Multiparametric tests should be conducted, detecting not only the IL-2, IL-4 and IFNγ, but also other cytokines secreted by the T cell, in order to closer analyse their differentiation.

5 CONCLUSION

Infections with human papillomaviruses are among the most common sexually transmitted infections, with peak incidence soon after the onset of sexual activity. Persistent infection with high-risk HPV types is considered to be a prerequisite in the development of virtually all invasive cervical cancers and their precursor lesions. Approximately two-thirds of all cervical cancers worldwide can be attributed to infection with HPV 16 and 18. Infections with low-risk HPV types, especially HPV 6 and 11, can cause benign lesions of the anogenital epithelia, also known as the genital warts.

Clinical trials of the two licenced prophylactic HPV vaccines, Gardasil® and Cervarix™, have shown remarkable efficacy against infection with, and genital disease caused by, the vaccine type HPV. In addition, both vaccines have shown evidence of cross-protection against homologous, non-vaccine HPV types. With respect to immunogenicity, clinical trials comparing directly the serological responses to the two vaccines have shown that Cervarix™ induces higher antibody titers than Gardasil®.

The study from SK Pacher showed that immunisation with both Gardasil or Cervarix induces memory T cell responses against vaccine type HPV as well as against homologous HPV types (HPV 31 and 45), with highest T cell frequencies being detected one month after the completion of the full vaccination cycle. Gardasil® induced higher T cell frequencies against the low-risk HPV 6 and 11, whereas Cervarix™ induced higher T cell frequencies against the high-risk HPV 16 and 18 as well as HPV 31 and 45. Measured 6 months after the third immunization, these differences in the cellular immune response between the two vaccines were not significant.

With help of the *ex vivo* assay this study analyses and compares the long-term memory T cell response in women immunized with Gardasil® or Cervarix™ on average 4.5 years prior to the study enrolment. High frequencies of memory CD4 T helper cells specific for the HPV L1 proteins from HPV 6, 11, 16 and 18 were detected in all 34 women included in the analysis at comparable levels to those detected 6 months after the third immunization.

In both the Gardasil® and the Cervarix™ group the highest T cell frequencies were detected against HPV 16 (followed by HPV 6>11>18 and HPV 6>18>11, respectively). In the Gardasil® group, HPV 6, 11 and 16 specific T cells were detected at comparable level, whereas the frequency of HPV 18-specific T cells was much lower. Surprisingly, the T cell responses to low-risk HPV types in the Cervarix™ group were detected at a comparable levels to the T cell

response against HPV 18. Direct comparison of the T cell responses showed no significant differences between the Gardasil® and the Cervarix™ group. A trend was observed for Gardasil® inducing stronger T cell responses against the low-risk HPV types, whereas Cervarix™ induced higher T cell frequencies against the high-risk HPV types.

The cross-reactivity against the phylogenetically related high-risk HPV types (HPV 31 and 45) was observed in both the Gardasil® and the Cervarix™ group. Gardasil® induced higher T cell frequencies against HPV 31, whereas stronger T cell response against HPV 45 was detected in the Cervarix™ group. Again, the memory T cells were detected at comparable levels to those detected 6 months after the third immunization.

Using the *in vitro* assay we succeeded in expanding and detecting the memory T cells at much higher frequencies compared to those detected using the *ex vivo* assay. In the Gardasil® group the strongest T cell response was detected against HPV 11 followed by HPV 6, 16 and 18, whereas highest T cell frequencies in the Cervarix™ group were detected against HPV 16, followed by HPV 11, 18 and 6. Surprisingly, in the Cervarix™ group the T cells against HPV 11 expanded better and were detected at higher frequencies than the T cells against HPV 18.

We were also able to expand *in vitro* the cross-reactive T cells against HPV 31 and 45. In the Cervarix™ group the T cell responses against HPV 31 and 45 were detected at up to 1.6-fold lower levels than those against HPV 16 and 18, respectively. Interestingly, in the Gardasil® group the memory T cells against HPV 31 and 45 were detected at higher mean frequencies than those against HPV 18.

Comparing the T cell frequencies detected using the *in vitro* assay, no significant differences were found between the Gardasil® and the Cervarix™ group. Gardasil® seemed to induce stronger T cell responses against HPV 6 and 45, whereas Cervarix™ induced higher T cell frequencies against HPV 16 and 18 as well as against HPV 11, although the HPV 11 VLPs are not included in the vaccine.

Analysing the expansion indices, we observed that the IFNγ positive T cells expand better *in vitro* than IL-2 positive ones. In the Gardasil® group the memory T cells against the low-risk HPV types expanded better than those against high-risk HPV types. The opposite was true for Cervarix™, the exception being the IFNγ-producing T cells against HPV 11 that showed equal expansion indices to HPV 16 and higher ones compared to HPV 18. Altogether the expansion

indices were higher in the Cervarix™ group compared to the Gardasil® group, the key likely lying in the adjuvant system AS04 used in Cervarix™.

Extrapolating the results from our *in vitro* assay, it is reasonable to assume that the *in vivo* exposure to vaccine type HPV and to homologous high-risk HPV 31 and 45 results in boosting of the vaccine-induced memory T cell responses. These responses are important for both the induction and maintenance of the humoral responses and might play a significant role in the outcome of the vaccination and the duration of protection against infection.

The highest antibody titers in the Gardasil® group were detected against HPV 11, followed by HPV 18, 16 and 6, whereas the strongest humoral immune response in the Cervarix™ group was detected against HPV 16, followed by HPV 18, 11 and 6. The antibody titers against the low-risk HPV types were up to 80-fold higher in the Gardasil® group, whereas the antibody titers against the high-risk HPV types were up to 5.6-fold higher in the Cervarix™ group. These differences were statistically highly significant. Both Gardasil® and Cervarix™ induced antibodies against HPV 31 at comparable levels, the antibody titers being 14- and 62-fold higher than the those against HPV 16, respectively.

We observed a lack of correlation between the cellular and the humoral immune responses detected in our study suggesting the heterogeneity in the immune response to vaccination between individuals. Since the Th2 cells are chiefly responsible for the activation of the B cells and their further differentiation into antibody secreting plasma cells, the correlation between the antigen-specific Th2 cell and antibody response is expected to be much more pronounced and should be tested by adequate methods.

In conclusion, high frequencies of memory CD4 T helper cells specific to HPV L1 antigens were detected up to 5.5 years after vaccination with either Gardasil® or Cervarix™ at comparable levels to those detected 6 months after the third immunization, the high cellular immune memory possibly portraying the long term efficacy of the two vaccines. On the polyclonal T cell level we were able to show a strong cross-reactivity with phylogenetically related HPV types.

6 ZUSAMMENFASSUNG

Humanen Papillomviren gehören zu den häufigsten sexuell übertragbaren Infektionen und zeigen einen Häufigkeitsgipfel bald nach dem Beginn sexueller Aktivität. Persistierende Infektionen mit den High-Risk-HPV Typen sind eine Voraussetzung für die Entwicklung von fast allen Zervixkarzinomen und deren Vorstufen. Etwa zwei Drittel aller Zervixkarzinome weltweit werden einer Infektion mit HPV 16 und 18 zugeschrieben. Low-Risk-HPV Typen, insbesondere HPV 6 und 11, können die benignen Läsionen der anogenitalen Epithelien, die genitalen Warzen, verursachen.

Klinische Studien mit den zwei zugelassenen prophylaktischen HPV Impfstoffen, Gardasil® und Cervarix™, haben eine Effektivität bis zu 100% gegen Infektionen sowie genitalen Erkrankungen durch die Impfstoff-HPV-Typen gezeigt. Kreuzprotektion gegen verwandten HPV-Typen wurde für beide Impfstoffe nachgewiesen. Bezüglich der Immunogenität konnten in klinischen Studien, die die serologische Antwort durch beide Impfstoffe direkt verglichen haben, höhere Antikörpertitern in Cervarix™-geimpften im Vergleich zu Gardasil®-geimpften Frauen ermittelt werden.

Die Daten von SK Pachert zeigten, dass eine Immunisierung mit Gardasil® bzw. Cervarix™ eine T-Zell-Gedächtnisantwort gegen die im jeweiligen Impfstoff enthaltenen HPV-Typen sowie verwandten HPV-Typen induziert. Beide Impfstoffe erzielten die höchsten T-Zell-Frequenzen einen Monat nach der dritten Impfung. Gardasil® induzierte höhere T-Zell-Frequenzen gegen die Low-Risk HPV 6 und 11, während Cervarix™ stärkere T-Zell-Antworten gegen die High-Risk HPV 16 und 18 sowie 31 und 45 induzierte. Diese Unterschiede in zellulären Immunantwort zwischen den beiden Impfstoffen glichen sich an und waren 6 Monate nach der dritten Impfung nicht signifikant.

Die vorliegende Arbeit analysiert und vergleicht mit Hilfe eines *ex-vivo*-Assays die Langzeit-T-Zell-Gedächtnisantworten in Frauen, die durchschnittlich 4,5 Jahren vor der Studienaufnahme mit Gardasil® bzw. Cervarix™ geimpft wurden. Hohe Frequenzen von HPV 6, 11, 16 und 18 L1 spezifischen CD4+ T-Helfer-Gedächtniszellen wurden in Blut von allen 34 in der Analyse eingeschlossen Frauen detektiert. Die Frequenzen waren vergleichbar mit entsprechenden Frequenzen, die 6 Monaten nach der dritten Impfung festgestellt wurden.

Sowohl in der Gardasil®- als auch in der Cervarix™-Gruppe wurden die höchsten T-Zell-Frequenzen gegen HPV 16 ermittelt (gefolgt von HPV 6>11>18 bzw. HPV 6>18>11). In der

Gardasil®-Gruppe wurden die HPV 6-, 11- und 16-spezifischen T-Zellen auf einem vergleichbar hohen Niveau gemessen, während die HPV 18-spezifische T-Zell-Antwort deutlich schwächer ausgeprägt war. Unerwarteterweise waren die T-Zell-Antworten gegen die Low-Risk-HPV Typen in der Cervarix™-Gruppe vergleichbar mit denjenigen gegen HPV 18. Der direkter Vergleich der T-Zell-Antworten zwischen der Gardasil®- und der Cervarix™-Gruppe ergab keine signifikanten Unterschiede. Ein Trend wurde beobachtet, in dem Gardasil® stärkere T-Zell-Antworten gegen die Low-Risk-HPV Typen hervorrieft, während Cervarix™ höhere T-Zell-Frequenzen gegen die High-Risk-HPV Typen induziert.

Die Kreuzreaktivität gegen verwandte High-Risk-HPV Typen (HPV 31 and 45) wurden sowohl in der Gardasil®- als auch in der Cervarix™-Gruppe beobachtet. Gardasil® induzierte höhere T-Zell-Frequenzen gegen HPV 31, während stärkere T-Zell-Antworten gegen HPV 45 in der Cervarix™-Gruppe festgestellt wurde. Die Frequenzen waren vergleichbar mit den entsprechenden Frequenzen, die 6 Monaten nach der dritten Impfung ermittelt wurden.

Unter Verwendung eines *in-vitro*-Assays gelang es, die T-Gedächtniszellen zu expandieren und deutlich höhere Frequenzen im Vergleich zu denjenigen, die mit Hilfe des *ex-vivo*-Assay gemessen wurden, zu ermitteln. In der Gardasil®-Gruppe wurde die stärkste T-Zell-Antwort gegen HPV 11 festgestellt (gefolgt von HPV 6>16>18), während die höchste T-Zell-Frequenzen in der Cervarix™-Gruppe gegen HPV 16 gemessen wurden (gefolgt von HPV 11>18>6). Unerwarteterweise konnten in der Cervarix™-Gruppe die T-Zellen gegen HPV 11 im Vergleich zu denjenigen gegen HPV 18 besser expandiert und auf höheren Frequenzen detektiert werden.

Die kreuzreaktive T-Gedächtniszellen gegen HPV 31 und 45 konnten auch *in vitro* expandiert werden. In der Cervarix™-Gruppe wurden bis zu 1,6-fach niedrigere Frequenzen von den T-Zellen gegen HPV 31 bzw. 45 im Vergleich zu denjenigen gegen HPV 16 bzw. 18 ermittelt. Interessanterweise wurden in der Gardasil®-Gruppe höhere Frequenzen von den T-Zellen gegen HPV 31 und 45 im Vergleich zu denjenigen gegen HPV 18 gemessen.

Zwischen der Gardasil®- und der Cervarix™-Gruppe wurden keine signifikante Unterschiede in der T-Zell-Antwort gefunden. Gardasil® induzierte tendenziell stärkere T-Zell-Antworten gegen HPV 6 und 45, während Cervarix™ höhere T-Zell-Frequenzen gegen HPV 16 und 18 sowie HPV 11 induzierte, trotz der Tatsache, dass HPV 11 VLPs nicht in dem Impfstoff enthalten sind.

Die *in-vitro*-Expansion von IFNγ+ T-Zellen war stärker als von IL-2+ T-Zellen, was ein Hinweis auf eine Th1-Antwort gibt. In der Gardasil®-Gruppe expandierten die T-Zellen gegen die Low-

Risk-HPV Typen besser als diejenigen gegen die High-Risk-HPV Typen. Das Gegenteil traf für Cervarix™ zu. Die einzige Ausnahme stellten die IFNγ+ T-Zellen gegen HPV 11 dar, die vergleichbar hohe Expansionsindizes mit HPV 16 und höhere Expansionsindizes als HPV 18 zeigten.

Extrapoliert man die Ergebnisse des *in-vitro*-Assays kann angenommen werden, dass eine *in-vivo*-Exposition gegen die Impfstoff-HPV Typen sowie gegen verwandte High-Risk HPV 31 und 45 die Impfstoff-induzierte T-Gedächtniszell-Antworten boosten könnte. Diese T-Zell-Antworten sind wichtig für die Induktion sowie Erhalt von den humoralen Immunantworten und spielen eventuell eine signifikante Rolle für den Erfolg der Immunisierung sowie die Dauer der Protektion gegen eine Infektion.

Die höchste Antikörpertiter in der Gardasil®-Gruppe wurden gegen HPV 11 ermittelt (gefolgt von HPV 18>16>6), während der stärkste humorale Immunantwort in der Cervarix™-Gruppe gegen HPV 16 gemessen wurde (gefolgt von HPV 18>11>6). Die Antikörpertiter gegen die Low-Risk-HPV Typen waren bis zu 80-fach höher in der Gardasil®-Gruppe, während Cervarix™ bis zu 5,6-fach höhere Antikörpertiter gegen den High-Risk-HPV Typen induzierte. Diese Unterschiede zwischen den zwei Gruppen waren statistisch hoch signifikant. Sowohl Gardasil® als auch Cervarix™ induzierten vergleichbar hohe Antikörpertiter gegen HPV 31. Diese waren 14- bzw. 62-fach niedriger als die Antikörpertiter gegen HPV 16.

Eine Heterogenität der Immunantwort auf die Impfung zwischen den einzelnen Personen wurde durch das Fehlen der Korrelation zwischen der zellulären und der humoralen Immunantwort nahegelegt. Da die Th2-Zellen hauptsächlich für die Aktivierung von B-Zellen sowie deren weiteren Differenzierung zu Antikörper-produzierenden Plasmazellen verantwortlich sind, ist eine ausgeprägtere Korrelation zwischen den Antigen-spezifischen Th2-Zellen und humoralen Immunantwort zu erwarten. Diese sollte mittels adäquater Methoden geprüft werden.

Zusammenfassend wurden hohe Frequenzen von HPV L1 spezifischen CD4+ T-Helfer-Gedächtniszellen bis zu 6 Jahren nach der Immunisierung mit Gardasil® und Cervarix™ gemessen. Diese waren vergleichbar mit denjenigen, die 6 Monate nach der dritten Impfung ermittelt wurden. Das ausgeprägte zelluläre Immungedächtnis spiegelt möglicherweise die Langzeiteffektivität der beiden Impfstoffe wider. Auf der polyklonalen T-Zell-Ebene konnten starke Kreuzreaktivitäten gegen verwandten HPV-Typen gezeigen werden.

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8 ABBREVIATION INDEX

AAHS	amorphous aluminium hydroxyphosphate sulphate
AIS	adenocarcinoma in-situ
APC	allophycocyanin
APC	antigen presenting cells
AS04	aluminium hydroxide plus 3-deacylated monophosphoryl lipid A
ASCUS	atypical squamous cells of undetermined significance
ATP	according-to-protocol
BCR	B cell receptor
BD	Becton Dickinson
BSA	bovine serum albumin
CA	condylomata acuminata
CC	cervical cancer
CD	cluster of differentiation
CIN	cervical intraepithelial neoplasia
CTL	cytotoxic T lymphocytes
DKFZ	germ. Deutsches Krebsforschungszentrum
DMSO	dimethylsulfoxid
DNA	deoxyribonucleic acid
E1-E7	early protein 1-7
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
EMBnet	European Molecular Biology Network
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
Fig.	figure
FITC	fluorescein isothiocyanate
FSC	forward scatter
FUTURE	Females United to Unilaterally Reduce Endo/Ectocervical disease
GMT	geometric mean titers
GSK	GlaxoSmithKline
H_0	null hypothesis
HIV	human immunodeficiency virus
HLA	human leukocyte antigen

HPV	human papillomavirus
HSIL	high-grade squamous intraepithelial lesions
IFN	interferon
IgG	immunoglobulin G
IL	interleukin
IQR	interquartile range
ITT	intention-to-treat
L1-L2	late protein 1-2
LCR	long control region
LSIL	low-grade squamous intraepithelial lesions
MHC I/II	major histocompatibility complex class I/II molecules
MPL	3-deacylated monophosphoryl lipid A
NR	not reported
p	p-value
Pap	Papanicolaou
PBMC	peripheral blood mononuclear cells
PBNA	pseudovirion-based neutralization assay
PBS	phosphate buffered saline
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
R1/2	region 1/2
RPMI	cell culture medium containing glucose, salts, aminoacids and vitamins
SD	standard deviation
SEB	staphylococcal enterotoxin B
SSC	side scatter
STD	sexually transmitted diseases
STIKO	Ständige Impfkommission am Robert-Koch-Institut
TCR	T cell receptor
Th1/2	T helper 1/2 cells
TLR	toll-like receptor
TNF	tumor necrosis factor
TVC	total-vaccinated-cohort
UR	upper right quadrant
VaC	vaginal cancer

VaIN	vaginal intraepithelial neoplasia
VC	vulval cancer
VIN	vulval intraepithelial neoplasia
VLP	virus-like particles
w/v	weight over volume

9 FIGURE INDEX

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11 ERKLÄRUNG

„Ich, Helena Maria Hepburn Drobnjak, erkläre, dass ich die vorgelegte Dissertation mit dem Thema: „Long term cellular immune response to prophylactic human papillomavirus (HPV) vaccines“ selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

Datum Unterschrift

12 LEBENSLAUF

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

13 PUBLIKATIONSLISTE

05.2011	Titel: „Long term ex vivo monitoring of memory CD4 T helper cell responses in women immunized with Gardasil® or Cervarix™", Eurogin 2011, Lisbon, Portugal (oral presentation)
05.2009	Titel: "Ex vivo monitoring of cellular immune responses in young women immunized with either Gardasil® or Cervarix™ four years prior to enrolment", 25th International Papillomavirus Conference 2009, Malmö, Schweden (Best Poster Award, oral presentation)
05.2009	Titel: „Nobelpreis für die Impfung gegen Zervixkrebs, aktuelle Daten und Leitlinienlage", Der Internist, Mai 2009; 50(5):617-626 von H.M. Hepburn, A.M.Kaufmann (PMID: 19384543)

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