Human papillomavirus vaccination induces neutralising antibodies in oral mucosal fluids

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Background: Mucosal human papillomaviruses (HPV) are a major cause of cancers and papillomas of the anogenital and oropharyngeal tract. HPV-vaccination elicits neutralising antibodies in sera and cervicovaginal secretions and protects uninfected individuals from persistent anogenital infection and associated diseases caused by the vaccine-targeted HPV types. Whether immunisation can prevent oropharyngeal infection and diseases and whether neutralising antibodies represent the correlate of protection, is still unclear.

Methods: We determined IgG and neutralising antibodies against low-risk HPV6 and high-risk HPV16/18 in sera and oral fluids from healthy females ($n = 20$) before and after quadrivalent HPV-vaccination and compared the results with non-vaccinated controls.

Results: HPV-vaccination induced type-specific antibodies in sera and oral fluids of the vaccinees. Importantly, the antibodies in oral fluids were capable of neutralising HPV pseudovirions *in vitro*, indicating protection from infection. The increased neutralising antibody levels against HPV16/18 in sera and oral fluids post-vaccination correlated significantly within an individual.

Conclusions: We provide experimental proof that HPV-vaccination elicits neutralising antibodies to the vaccine-targeted types in oral fluids. Hence, immunisation may confer direct protection against type-specific HPV infection and associated diseases of the oropharyngeal tract. Measurement of antibodies in oral fluids represents a suitable tool to assess vaccine-induced protection within the mucosal milieu of the orophayrynx.

Human papillomaviruses (HPV) are small, double-stranded DNA viruses and, to date, more than 180 HPV (geno-)types have been identified (Bernard *et al*, 2010). These epitheliotropic viruses exclusively infect epithelial cells of skin or mucosa, allowing for their classification into cutaneous or mucosal HPV types, respectively. Depending on the virus type and host, HPV infection can be asymptomatic, can induce benign warts or papillomas, or lead to the development of anogenital malignancies—most notably cervical carcinomas—and head and neck cancers (HNSCC).

HNSCC represent a rising global burden with annually more than 500 000 incident cases and 300 000 deaths (Marur *et al*, 2010; Ferlay *et al*, 2015). These malignancies, predominantly squamous cell carcinomas, which arise from the mucosa of the oral cavity, the oro-, hypo-, nasopharnx, the larynx, and the sinonasal tract, can be

divided into two distinct subsets according to their aetiology. The first subset is firmly associated with heavy tobacco and/or alcohol consumption, poor oral hygiene, or predisposing genetics (but is HPV-negative). In the past decade, a second entity was recognised as caused by infection with mucosal high-risk HPV, independently of the established risk factors (D'Souza *et al*, 2007; Gillison *et al*, 2008; Mehanna *et al*, 2013). In the latter the rate of detection of HPV DNA in the tumours overall was variable between the different anatomical sites, with the highest rate observed in cancers of the oropharynx (45.8%; in particular of the tonsils and the base of tongue at 53.9%), the oral cavity (24.2%), and the larynx/ hypopharynx (22.1%) (Ndiaye *et al*, 2014). The DNA was localised in the nuclei of cancerous cells at all stages—in preinvasive/ invasive tumours and metastases—in integrated form and found to

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be transcriptionally active (Steenbergen et al, 1995; Gillison et al, 2000). The most dominant causative HPV type is mucosal high-risk HPV 16, which accounts for >90% of the carcinomas (Gillison et al, 2000; Kreimer et al, 2005; D'Souza et al, 2007; Marur et al, 2010). Second, high-risk HPV 18, and in rare cases other high-risk mucosal types and low-risk mucosal HPV 6 and 11 were detected in the HNSCC (Gillison et al, 2000; Kreimer et al, 2005). However, the latter two types predominantly cause papillomas of the respiratory and the laryngeal tract, including recurrent respiratory papillomatosis, a rare disease in children and adults that due to extensive warty growths in the upper airway and dissemination to bronchi, may cause significant airway obstruction and may undergo malignant transformation (Venkatesan et al, 2012; Taliercio et al, 2015).

In contrast to the well-established pathogenesis of anogenital, in particular cervical, HPV infection and associated malignancies, the natural history of HPV infection in the oropharyngeal tract is not as extensively investigated. Transmission of the virus most likely occurs via orogenital contact and oral HPV DNA prevalence was consistently associated with sexual behaviour. For instance, patients with HPV-positive oropharyngeal cancers were shown to have a higher number of lifetime sexual partners (as surrogate for oral HPV exposure) and presented oral HPV infection more frequently than controls (D'Souza et al, 2007; Heck et al, 2010). In the general population, oral HPV was reported at 4.5–6.9% and the prevalence of infections with one of the mucosal high-risk HPV types given at 3.5–3.7%, of which HPV 16 accounted for 1–1.3% (Kreimer et al, 2010; Chung et al, 2014).

Prophylactic HPV vaccination is highly immunogenic, leading to seroconversion in nearly all immunised individuals. In previously uninfected individuals, administration of the HPV vaccines prevented persistent infection and associated diseases of the anogenital tract that are caused by the HPV types included in the vaccine formulation with an efficacy of up to 100% (De Vincenzo et al, 2014; Herrero et al, 2015). This was convincingly demonstrated in multiple large, randomised, controlled, clinical trials and resulted in licensing and implementation of the vaccines in numerous national immunisation programmes (Markowitz et al, 2014; WHO, 2014). For vaccine-induced protection both, humoral and cellular, immune mechanisms are responsible. After vaccination, HPV type-specific antibodies capable of neutralising infectious virions were detectable in sera—at up to 4-log higher levels compared with titres induced by natural infection—and in cervicovaginal secretions (Harper et al, 2006; Schwarz et al, 2010; Petäjä et al, 2011). In addition, cell-mediated immune responses to the viral proteins are equally important for the success of prophylactic vaccination. Whereas T-helper responses are critically involved in the generation and maintenance of protective B-cell responses and the induction of neutralising antibodies, cytotoxic T-lymphocyte responses to the viral proteins are important for elimination of HPV-infected cells that escaped antibody neutralisation. While vaccine efficacy is firmly established for the prevention of anogenital infection and diseases, studies on the impact of vaccination on oral HPV infection and HPV-related oropharyngeal diseases are sparse.

First evidence for a reduction of oral HPV infection after vaccination was obtained from the Costa Rica vaccine trial (Herrero et al, 2013). Prevalence of oral HPV 16/18 DNA was reduced in the recipients of the bivalent HPV vaccine 4 years post-vaccination compared with hepatitis A-vaccinated controls, with vaccine efficacy against HPV 16/18 infection estimated at 93.3% in this large cohort. Although this important study strongly indicates that HPV vaccination has the potential to confer protection against oral infection with the vaccine-targeted types, data on the baseline HPV prevalence are lacking and information on the presence of HPV-specific antibodies in the oral fluids of the vaccinees and their protective capacity is not available.

In this study, we determined the presence of type-specific IgG and neutralising antibodies in oral fluids after immunisation with the quadrivalent HPV vaccine and investigated a possible correlation between (neutralising) antibody titres in paired serum and oral fluids. We furthermore aimed at providing experimental proof that antibodies isolated from oral fluids are capable of preventing HPV infection, thus giving insight into one possible mechanism how oral HPV infection and possibly associated diseases can be prevented by systemic administration of an HPV vaccine.

MATERIAL AND METHODS

Study population. The study protocol was approved by the ethics committee of the Medical University of Vienna before initiation (EK 690/2008) and informed consent was obtained from each participant.

Study participants ($n = 34$) were healthy females between 18 and 26 years of age (mean age 23.2 years) with four or less lifetime sexual partners and no history of anogenital HPV infection, including condylomata and cervical dysplasia. Recipients of immunoglobulins, blood products, and immunomodifying drugs within 6 months before study initiation or pregnant or nursing women were excluded. Participants were recruited into two groups: 20 participants (age range 19–24 years, mean age 22.6 years) received the quadrivalent HPV vaccine against low-risk HPV 6/11 and high-risk HPV 16/18 intramuscularly according to the licensed schedule; 14 participants (age range 22–25 years, mean age 23.9 years) were included in the control group and not vaccinated. Medical and sexual histories were obtained at enrolment. All participants completed the compulsory four visits within the prespecified intervals.

Sample collection. Serum and oral fluids were collected from all participants before immunisation/study initiation (month 0), at each subsequent vaccination (months 2 and 6), and four weeks after the final immunisation/at end of study (month 7). Oral fluids were collected by inserting two pads (OraSure Technologies Inc., PA, USA) between buccal mucosa and gingiva for 4 min. To analyse the presence of HPV-specific total IgG antibodies by ELISA, one pad was subsequently inserted into the supplied vial (OraSure) containing a transportation buffer with preservative. To extract the oral fluid, vials were centrifuged at 2400 **g** for 20 min, the pad removed, and the remaining liquid collected. For detection of HPV-specific neutralising antibodies by pseudovirion (PsV)-based neutralisation assays, the second collection pad was repeatedly rinsed with 1 ml of phosphate-buffered saline (PBS) and the eluate collected by centrifugation at 2400 **g** for 15 min. This procedure was repeated with another millilitre of PBS, eluates were pooled and subjected to sterile filtration using Corning Costar Spin-X centrifuge tube filters with a 0.2-μm pore filter size at 13 000 r.p.m. for 10 min. Both, sera and oral fluids, were investigated for the presence of HPV-specific antibodies. HPV 6 served as representative for low-risk, HPV 16 and HPV 18 for high-risk mucosal HPV. All three types are targeted by the quadrivalent HPV vaccine.

Virus-like particle–ELISA. Recombinant baculoviruses expressing the major capsid protein L1 of HPV 6, 16, and 18 were described previously (Kirnbauer et al, 1993; Rose et al, 1994) and virus-like particles (VLP) of the respective HPV types generated and purified by standard methods (Kirnbauer et al, 1994; Handisurya et al, 2010). Sera and oral fluids were tested by VLP-ELISA for the presence of HPV-specific IgG antibodies to HPV types 6, 16 and 18, as described previously (Handisurya et al, 2010). Sera were fourfold serially diluted in PBS, ranging from 1:1000 to 1:64 000, oral fluid samples were used in dilutions of

1:10 and 1:100 (in PBS). Polyclonal rabbit sera, obtained after immunisation with VLP of the respective HPV types—either HPV 6, HPV 16, or HPV 18—served as positive controls, VLP-coated wells without addition of sample (PBS-only wells) as negative control in the assays. Optical density (OD) values were determined at 405 nm test wavelength/490 nm reference wavelength. Seropositivity was defined as mean OD values higher than twice the mean values of pooled sera obtained from non-infected, sexually naive controls.

Pseudovirion-based neutralisation assays. PsV of HPV types 6, 16, and 18 were generated by co-transfection of the human embryonic kidney cell line 293TT with a plasmid encoding for the respective viral capsid genes and a reporter plasmid encoding for secreted alkaline phosphatase (SEAP), according to standard protocols (Buck et al, 2005; Handisurya et al, 2010). For analysis by Pseudovirion-based neutralisation assays (PBNA), participants' sera were fourfold serially diluted from 1:400 to 1:25 600, oral fluids from 1:4 to 1:16, and both were independently tested for their ability to inhibit infection of 293TT cells by PsV of either HPV type 6, 16, or 18, as previously published. Rabbit polyclonal sera raised against either HPV 6, HPV 16, or HPV 18 VLP or a mixture of the analysed PsV and media without samples (PBS-only wells) were used as positive or negative controls, respectively. OD values were measured at 405 nm, as given in the manufacturer's instructions. Samples showing at least 50% reduction in SEAP activity were considered neutralising.

Statistical analyses. Statistical analyses were performed using the Statistical Package for the Social Sciences Version 15.0. (SPSS Inc., Chicago, IL, USA), P-values <0.05 (two-sided asymptotic significance) were considered statistically significant. Differences between the vaccinated and the non-vaccinated cohorts were calculated using the Mann–Whitney U-test, differences between samples obtained before and one month after completion of the vaccination series from the same study participant employing the Wilcoxon signed-rank test. Correlations between overall HPV-specific IgG antibody concentrations and neutralising antibodies against the same HPV type in samples obtained post-vaccination were determined using the Pearson's χ^2-test.

RESULTS

HPV immunisation induced total IgG and neutralising antibodies to the vaccine-targeted types in sera of the vaccinees.

Sera of all study participants were analysed for the presence of type-specific IgG and neutralising antibodies against HPV 6, HPV 16 and HPV 18 by VLP-ELISA and PBNA, respectively. These three types are the most common and important representatives of low- and high-risk mucosal HPV and are included in the quadrivalent vaccine formulation.

At month 7 (1 month after the third and final immunisation of the vaccination cohort) all participants of the vaccine group had seroconverted, showing high titres ranging from 4000 to 64 000 for HPV 6, 4000 to 64 000 for HPV 16, and 1000 to 64 000 for HPV 18 (Figure 1A–C). Compared with the baseline levels (at month 0) of the total study cohort and to the results obtained in controls at month 7, the elevation of immunisation-induced IgG antibodies in sera of vaccinees against the three tested HPV types was statistically significant ($P<0.001$ for all; Figure 1A–C).

Neutralising antibodies are thought to represent the correlate of protection against HPV infection. In the vaccination cohort, neutralising antibodies against HPV 6 were detected in 75% (15/20) of the participants (titres ranging from 400 to 1600), anti-HPV 16 neutralising antibodies in all vaccinees (titres ranging from 1600 to 6400), and anti-HPV 18 neutralising antibodies in 95% (19/20) of the vaccinated participants (titres ranging from 400 to 6400) at month 7. The neutralising antibody titres of all three types were significantly higher in the vaccinees than the baseline titres (at month 0) of the total study cohort ($P<0.001$; Figure 2A–C). However, at study enrolment neutralising antibodies against HPV 6 were found in two study participants, both with titres of 1600, (Figure 2A). The two individuals belonged to the vaccine cohort and after vaccination the titres remained positive at the same dilution. Surprisingly, overall IgG against HPV 6, as determined by VLP-ELISA, were not detectable in these particular subjects at baseline and post-vaccination the anti-HPV 6 IgG levels increased to titres of 64 000. At month 7 type-specific neutralising antibodies to HPV 6, 16, and 18 in the sera of the vaccinated group were significantly elevated compared with non-immunised controls ($P<0.001$ for all three HPV types tested; Figure 2A–C). The type-specific levels of total IgG and neutralising antibodies in sera showed a significant correlation ($P<0.001$) for each tested HPV type, 6, 16, and 18 (not shown).

After vaccination type-specific IgG antibodies to the vaccine types were detectable in oral fluids. To determine whether type-specific IgG antibodies are elicited after vaccination in oral fluids, samples were analysed by ELISA using VLP of HPV 6, 16, and 18 as the antigen.
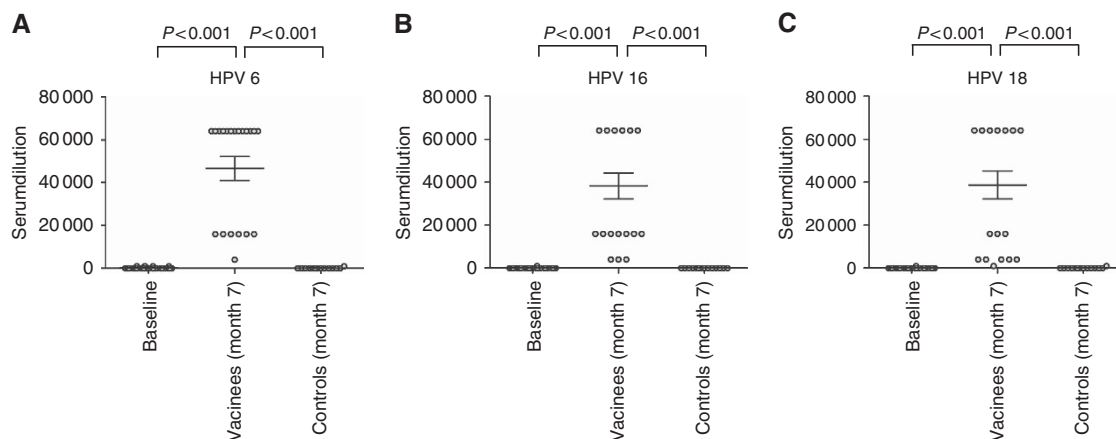

Figure 1. Total HPV-specific IgG antibodies in sera of the study cohort. IgG antibodies to (**A**) HPV 6, (**B**) HPV 16, and (**C**) HPV 18 were determined by virus-like particle ELISA at month 0 (baseline) in all study participants ($n=34$) and at month 7 in the vaccine ($n=20$) and control ($n=14$) cohort. The scatter plots display the serumdilutions, which gave a positive signal. The horizontal bars show the mean value for each group ± s.d.
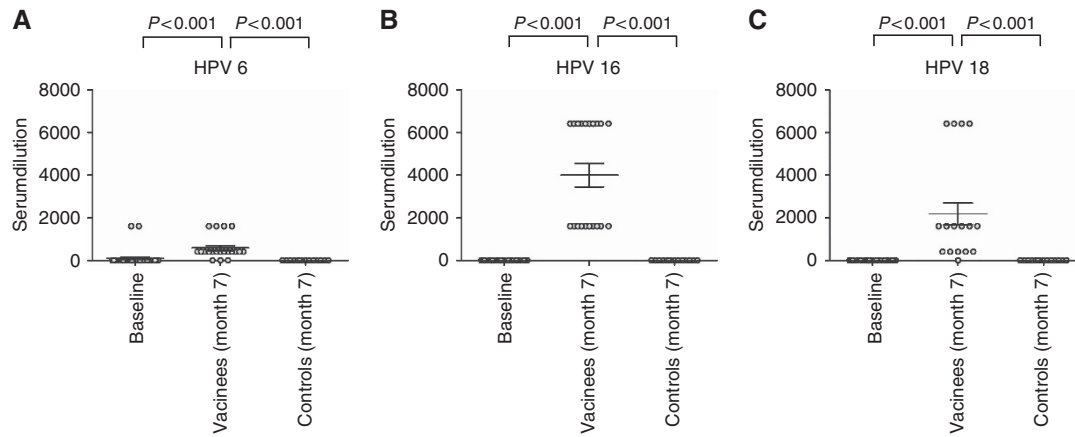
Figure 2. Neutralising serum antibodies in sera of the study participants. Pseudovirion-based neutralisation assays were employed to determine the presence of neutralising antibodies to (A) HPV 6, (B) HPV 16, and (C) HPV 18 in the study cohort. Neutralising antibodies were evaluated in sera obtained at month 0 (baseline) from the total cohort ($n = 34$) and at month 7 from the vaccinees ($n = 20$) and the controls ($n = 14$). The scatter plots display the serumdilutions, which gave a positive signal. The horizontal bars show the mean value for each group $\pm$ s.d.
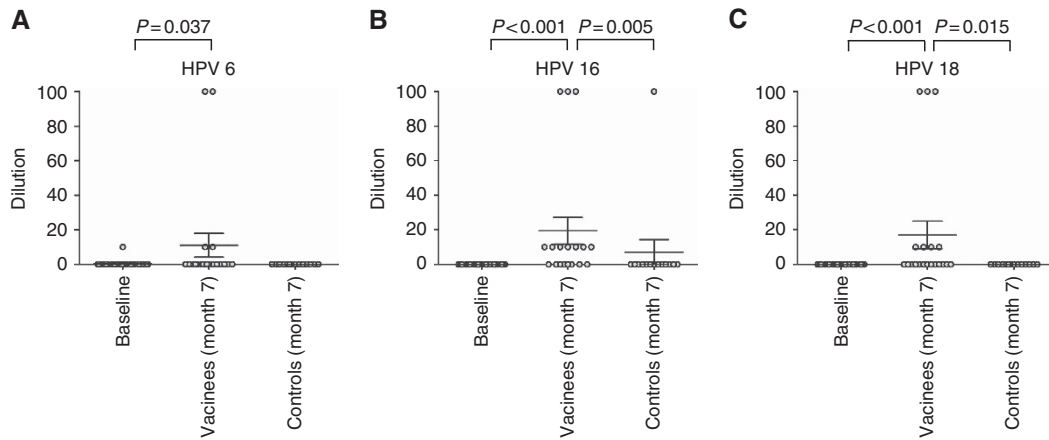

Figure 3. Total HPV-specific IgG antibodies in oral fluids of the study cohort. By virus-like particle ELISA IgG antibodies to (A) HPV 6, (B) HPV 16, and (C) HPV 18 were detected in oral fluids. Oral fluid samples were obtained at month 0 (baseline; $n = 34$) and at month 7 from the vaccine ($n = 20$) and the control ($n = 14$) groups. The plots display the dilutions of the oral fluids, at which a positive signal was obtained. The horizontal bars show the mean value for each group $\pm$ s.d.

At month 0, antibodies against HPV 6 were found in one participant at a dilution of 10. All other participants did not reveal detectable antibodies in the oral fluids to any of the tested types at this time point. One month after completion of the vaccination series, antibodies against HPV 6 were found in 15% (3/20) of the vaccinees at dilutions of 10 to 100. The one individual that had detectable IgG against HPV 6 at baseline belonged to this cohort and remained anti-HPV 6 positive at the same dilution after vaccination. In the vaccine recipients IgG antibodies against HPV 16 were detectable in a majority (60%; 12/20) and against HPV 18 in 35% (7/20) with detectable titres ranging from 10 to 100. Compared with baseline, titres were statistically significantly elevated at month 7 ($P = 0.037$ for HPV 6, $P < 0.001$ for HPV 16, $P < 0.001$ for HPV 18; Figure 3A–C). In the control arm, one participant had antibodies against HPV 16 at a dilution of 100 at the 7-month time point. Neither anti-HPV 6 nor anti-HPV 18 antibodies were found in this individual. All other controls remained negative to the tested HPV types 6, 16, and 18 in the oral fluids throughout the study period (Figure 3A–C). HPV-specific IgG antibody levels in oral fluids of the vaccine group were significantly higher for HPV 16 ($P = 0.005$) and HPV 18 ($P = 0.015$) compared with the non-vaccinated group. Although antibody titres for HPV 6 were increased in vaccinated compared

with non-vaccinated participants, differences were not statistically significant ($P = 0.080$).

Anti-HPV 16 IgG antibody levels in sera and oral fluids after vaccination are significantly correlated. Next, we compared the levels of IgG antibodies in sera and oral fluids against the same HPV type within the same vaccinated individual to determine whether evaluation of antibodies in oral fluids can be used as surrogate for measuring serum antibody titres. No statistically significant correlations were found for HPV 6 ($P = 0.052$) and HPV 18 ($P = 0.418$). However, this may be due to the low numbers of positives, especially when results were obtained from oral fluids. In contrast, anti-HPV 16 IgG antibodies in sera and in oral fluids correlated significantly ($P = 0.042$; Table 1).

HPV-specific antibodies present in oral fluids after HPV vaccination were neutralising. The important correlate for protection against HPV infection are thought to be neutralising antibodies, thus the proportion of neutralising antibodies in oral fluids were determined using PBNA assays. Of note, in contrast to serum samples, to our knowledge, these assays are not validated for oral fluids.

Neutralising antibody titres specific for HPV 6, 16 or 18 in the oral fluids were determined in vaccinees and controls at months 0

and 7, respectively. Both samples of an individual were analysed within the same experiment and on the same plate. To rule out any possible bias due to factors such as individual differences in composition or viscosity of oral fluids, results are given as differences in the titres between months 0 and 7 (Δ titre steps). In the vaccine cohort, 35% (7/20) of participants showed a one-step increase in neutralisation titres to HPV 6 after completion of the immunisation series compared with baseline. For HPV 16, 55% (11/20) of the vaccinees had an increase of one titre step at month 7. The highest increase of two titre steps were observed in 10% (2/20) of the vaccinated participants against HPV 18. A one-step increase against the same type 18 was noted for 30% (6/20) of this cohort. In the control population neutralising antibody titres were not elevated after the 7-month study period to any tested HPV types, with the exception of one participant, who had a one-step increase in the anti-HPV 18 neutralising titres at month 7 compared with baseline. However, anti-HPV 18 IgG were undetectable by VLP-ELISA in the oral fluid of this individual at any time point tested. Surprisingly, a decrease in the titres was noted for some study participants. In the vaccine population decreased titres were observed in 10% (2/20) for HPV 6, 5% (1/20) for HPV 16, and 5% (1/20) for HPV 18, and in the non-vaccinated population in 21.4% (3/14) for HPV 6 and HPV 16 and 35.7% (5/14) for HPV 18. Although the relevance of the observed decrease in titre steps is unclear, nevertheless, the negative differences were included in subsequent statistical calculations. Analyses on the differences in titre steps of the neutralising antibodies in oral fluids between the vaccine and the control groups at month 7 revealed statistically significant differences between changes in titre steps for all three tested types. For vaccinees the increase in the titres steps at month 7 was significantly higher compared with controls with $P = 0.026$ for HPV 6, $P = 0.001$ for HPV 16, and $P = 0.009$ for HPV 18 (Figure 4A–C).

We next determined the number of immunisations that were necessary to elicit detectable neutralising antibodies in the oral fluids. However, to date the threshold of antibody titres that are sufficient for protection is not known. As prime example we chose high-risk HPV 16, as this type induces a robust immune response and represents the most important virus type. In our population, the majority (63%; 7/11) mounted detectable anti-HPV 16 neutralising antibodies in the oral fluids only after the third and final vaccination (data not shown).

The increased titres of neutralising antibodies against high-risk HPV 16 found in sera and oral fluids of vaccinees at month 7 correlated significantly. We next investigated whether the HPV-vaccine-induced neutralising antibodies in sera correlated with neutralising antibodies in oral fluids within the same individual. As mentioned above, we regarded the differences in titre steps (Δ titre steps) of neutralising antibodies in oral fluids as more relevant than the absolute titres. Therefore, to allow for an appropriate comparison, we additionally determined the increases in titre steps for neutralising antibodies in sera. Similar to the results of type-specific total IgG antibodies between sera and oral fluids, no statistically significant correlations could be observed between neutralising antibody titres of sera and oral fluids for HPV 6 and HPV 18 ($P = 0.883$ and $P = 0.806$, respectively). In contrast, for high-risk mucosal HPV 16 the differences in titre steps of neutralising antibodies correlated significantly between corresponding sera and oral fluids ($P = 0.023$) (Table 2).

Table 1. Summary of IgG antibodies by virus-like particle ELISA against HPV 6, HPV 16, and HPV 18 in paired sera and oral fluids after HPV vaccination

Serum titre	Vaccinees oral fluid titre				Controls oral fluid titre			
	100	10	0	Total	100	10	0	Total
HPV 6								
64 000	0	1	12	13	0	0	0	0
16 000	2	1	3	6	0	0	0	0
4000	0	0	1	1	0	0	0	0
1000	0	0	0	0	0	0	1	1
0	0	0	0	0	0	0	13	13
Total	2	2	16	20	0	0	14	14
HPV 16								
64 000	1	5	4	10	0	0	0	0
16 000	1	3	3	7	0	0	0	0
4000	2	0	1	3	0	0	0	0
1000	0	0	0	0	0	0	0	0
0	0	0	0	0	1	0	13	14
Total	4	8	8	20	1	0	13	14
HPV 18								
64 000	0	5	7	12	0	0	0	0
16 000	0	0	3	3	0	0	0	0
4000	0	0	4	4	0	0	0	0
1000	0	0	1	1	0	0	1	1
0	0	5	0	0	0	0	14	14
Total	0	5	15	20	0	0	14	14

DISCUSSION

In this study, we found that HPV-vaccination induced specific antibodies to the vaccine-targeted types in blood and oral fluids.
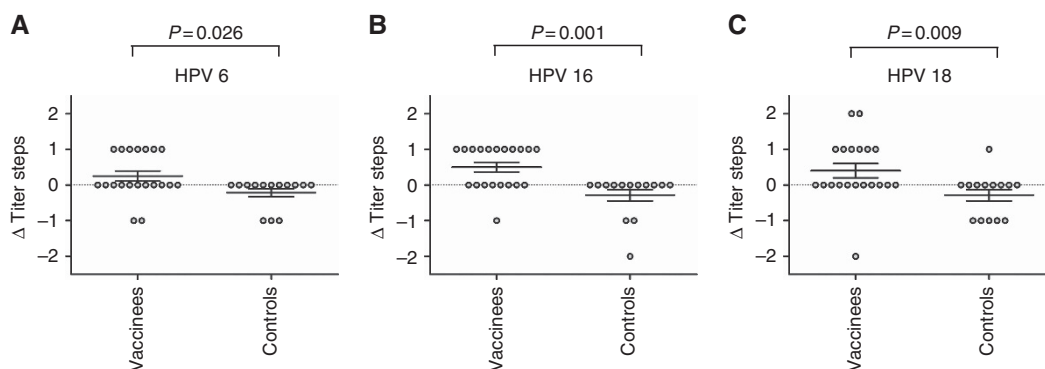

Figure 4. Differences in neutralising antibody titres (Δ titre steps) in oral fluids between vaccinated and non-vaccinated participants. Neutralising antibody titres to (**A**) HPV 6, (**B**) HPV 16, and (**C**) HPV 18 were measured by pseudovirion-based neutralisation assays in oral fluids in all participants ($n = 34$) at month 0 and month 7. The differences in titres between these time points are given as Δ titre steps. The plots display the Δ titre steps of the neutralising antibodies in oral fluids of the vaccinees and the controls. The horizontal bars show the mean value for each group ± s.d.

Table 2. Summary of differences in titre steps (Δ titre steps) of neutralising antibodies against HPV 6, HPV 16, and HPV 18 in paired sera and oral fluids after HPV vaccination

	Vaccinees Δ titre steps (oral fluids)				Controls Δ titre steps (oral fluids)			
HPV 6								
Δ Titre steps (sera)	1	0	−1	Total	1	0	−1	Total
0	2	0	0	2	0	11	3	14
1	4	7	2	13	0	0	0	0
2	1	1	0	2	0	0	0	0
3	0	3	0	3	0	0	1	1
Total	7	11	2	20	0	11	3	14
HPV 16								
Δ Titre steps (sera)	2	1	0	Total	2	1	0	Total
0	0	0	0	0	0	0	14	14
1	0	0	0	0	0	0	0	0
2	0	3	7	10	0	0	0	0
3	0	8	2	10	0	0	0	0
Total	0	11	9	20	0	0	14	14
HPV 18								
Δ Titre steps (sera)	2	1	0	Total	2	1	0	Total
0	0	0	1	1	0	1	13	14
1	1	1	3	5	0	0	0	0
2	0	4	6	10	0	0	0	0
3	1	1	2	4	0	0	1	1
Total	2	6	12	20	0	1	13	14

Importantly, we demonstrated that the elicited antibodies in oral fluids were capable of neutralising infectious PsV of the homologous HPV type *in vitro* and therefore may represent one protective correlate against infection of the oropharyngeal tract with the vaccine-targeted mucosal HPV types. Furthermore, we showed that the increased titres of neutralising antibodies against high-risk HPV 16—which causes the vast majority of HPV-attributable HNSCC—correlated significantly between sera and oral fluids of vaccinees. Therefore, (non-invasive) measurements of antibodies in oral fluids can be used in the future to assess vaccine-induced protection within the mucosal milieu of the orophayrynx, at least for this important type.

Equivalent work in the female genital tract has been published previously, demonstrating HPV-specific antibodies in sera and at the disease-relevant sites of the anogenital tract. HPV 16 and 18 antibodies were detected in cervicovaginal secretions of vaccinated girls and women and the measured levels strongly correlated with the respective titres in sera (Kemp *et al*, 2008; Schwarz *et al*, 2010; Petäjä *et al*, 2011).

The available prophylactic HPV vaccines are safe and highly efficacious in preventing persistent infection and associated diseases of the anogenital tract, caused by the vaccine-targeted HPV types. So far, two mechanisms seem to be responsible for the local protective effects at the infection sites by the circulating antibodies that are elicited by intramuscular immunisation. First, the circulating antibodies are thought to transudate from sera into the cervical mucus and, second, to exudate at sites of microtrauma or abrasions, which are a prerequisite for infection, into the surrounding tissues (Roden and Wu, 2006; Kemp *et al*, 2008). Albeit conceivable, so far it was not clear whether the protective mechanism(s) established for the anogenital tract are also applicable to HPV-induced diseases of the oropharynx. IgG antibodies against HPV 6, 11, and 16 were reported to be present in oral fluids of HIV-positive participants for whom the genital HPV status was unknown and in women with cervical dysplasia and carcinomas (Marais *et al*, 2001; Cameron *et al*, 2003; Marais *et al*, 2006). While after natural HPV infection antibodies are induced in about 70–80% of individuals with generally low and not

very robust titres (Ho *et al*, 1998), the serological response after vaccination is up to four logs higher. Although the presence of HPV-specific IgG antibodies was reported in oral fluids after parenteral immunisation of a large cohort, the functionality, that is, the neutralisation potential, of the detected antibodies was unknown (Rowhani-Rahbar *et al*, 2009). Herein, we provide experimental proof for the presence of HPV-specific neutralising antibodies that are elicited by vaccination at oral sites and in oral fluids in humans and thus may be capable of preventing persisting oral HPV infection, similar to the anogenital site.

Oral fluid represents the liquid in the oral cavity, which consists of saliva—secreted by the salivary glands—and oral mucosal transudate, the serous liquid that, originating from the capillaries, passes the buccal and gingival mucosa to enter the mouth. Two major antibody classes are present in oral fluids: secretory IgA and IgG. Whereas IgA is synthesised by plasma cells in salivary glands, most IgG are derived from serum, although some IgG is locally produced. The translocation of IgG from blood to extracellular fluid occurs most notably in the dental capillary bed. Systemic vaccine administration strongly induces serum IgG antibodies, however, generally poorly stimulates secretory IgA at mucosal sites, as shown for instance for influenza (Moldoveanu *et al*, 1995; Belshe *et al*, 2000). As in this study we addressed the question whether intramuscular immunisation elicits neutralising IgG antibodies in sera and oral fluids, we focused on the detection of IgG antibodies. Evaluation of HPV-specific IgA in oral fluid to the vaccine-targeted types will be of interest for future investigations.

This study was not designed to examine whether elicited neutralising antibodies in the oral fluids can effectively prevent persistent infection and/or the development of oropharyngeal HPV-associated diseases in our study population. However, prior epidemiological studies provided evidence that HPV vaccination leads to a decrease in oral HPV infection (Herrero *et al*, 2013; Grün *et al*, 2015). Albeit limited by the small sample size of the study population, the findings presented herein indicate one possible mechanism how systemic HPV vaccination may exert protection from oral infection. In addition, owing to the fact that oropharyngeal HPV infection is largely associated with sexual behaviour, the decrease in the prevalence of anogenital HPV infection due to vaccination might indirectly reduce the incidence of oral HPV infection. This indicates the feasibility of HPV vaccination for protection against oropharyngeal HPV infection and associated diseases.

In addition to humoral immune responses, T-cell responses contribute to the overall protection against HPV infection and diseases. Vaccination-induced CD8^{+}-driven T-cell response may represent the key mechanism whereby virus-infected cells are eliminated from both the oral cavity and the anogenital tract, presumably before clinical lesions are established. This is supported by previous observations on vaccinees who remained protected against HPV infection after waning of detectable antibody titres (Joura *et al*, 2008). To this end, L1-specific T-cell responses were measured after vaccination by proliferation of both CD4^{+} and CD8^{+} T cells and efficient cytokine responses inducing both, Th1 and Th2, patterns of cellular immune responses were detected in the vaccinees (Pinto *et al*, 2003; Pinto *et al*, 2005; Weinberg *et al*, 2012). However, further studies to elucidate the role of cytotoxic T cells in mucosal HPV (re)infection need to be performed.

ACKNOWLEDGEMENTS

CONFLICT OF INTEREST

RK is a coinventor on HPV16 L1 patents licensed to GlaxoSmithKline (GSK) and Merck (MSD). The remaining authors declare no conflict of interest.

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