

## RESEARCH ARTICLE

# Effect of the bivalent HPV vaccine on viral load of vaccine and non-vaccine HPV types in incident clearing and persistent infections in young Dutch females

Pascal van der Weele<sup>1,2\*</sup>, Martijn Breeuwsma<sup>1</sup>, Robine Donken<sup>1,2<sup>aa</sup>ab</sup>, Elske van Logchem<sup>1</sup>, Naomi van Marm-Wattimena<sup>1</sup>, Hester de Melker<sup>1</sup>, Chris J. L. M. Meijer<sup>2</sup>, Audrey J. King<sup>1</sup>

**1** National Institute for Public Health and the Environment (RIVM), Centre for Infectious Disease Control, Bilthoven, the Netherlands, **2** Vrije Universiteit - University Medical Center (VUmc), Department of Pathology, Amsterdam, the Netherlands

<sup>aa</sup> Current address: University of British Columbia, BC Children's Hospital Research Institute, Vancouver, British Columbia, Canada

<sup>ab</sup> Current address: University of British Columbia, BC Women's Health Research Institute, Vancouver, British Columbia, Canada

\* [pascalvanderweele@gmail.com](mailto:pascalvanderweele@gmail.com)



## OPEN ACCESS

**Citation:** van der Weele P, Breeuwsma M, Donken R, van Logchem E, van Marm-Wattimena N, de Melker H, et al. (2019) Effect of the bivalent HPV vaccine on viral load of vaccine and non-vaccine HPV types in incident clearing and persistent infections in young Dutch females. *PLoS ONE* 14 (3): e0212927. <https://doi.org/10.1371/journal.pone.0212927>

**Editor:** Magdalena Grce, Rudjer Boskovic Institute, CROATIA

**Received:** September 6, 2018

**Accepted:** February 12, 2019

**Published:** March 4, 2019

**Copyright:** © 2019 van der Weele et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Data Availability Statement:** All relevant data are within the manuscript and its Supporting Information files.

**Funding:** This work was supported by the Ministry of Health, Welfare and Sports, the Netherlands. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## Abstract

### Background

HPV vaccination with the bivalent vaccine is efficacious against HPV16 and 18 infections and cross-protection against non-vaccine HPV types has been demonstrated. Here, we assessed (cross-) protective effects of the bivalent HPV16/18 vaccine on incident and persistent infections and viral load (VL) of fifteen HPV types in an observational cohort study monitoring HPV vaccine effects.

### Methods

Vaginal samples were obtained annually. Type-specific VL assays were developed for HPV6,11,31,33,35,39,45,51,52,56,58,59 and 66 and used in addition to existing HPV16 and 18 assays. Rate differences of incident clearing and persistent infections were correlated with differences in VL and vaccination status.

### Results

HPV16/18 vaccination resulted in significantly lower incidence of HPV16/18 infections and significantly lower VL in breakthrough HPV16 ( $p < 0.01$ ) and 18 infections ( $p < 0.01$ ). The effects of vaccination on non-vaccine type VL were ambiguous. Incidence and/or persistence rates of HPV31, 33, 35 and 45 were reduced in the vaccinated group. However, no significant type specific VL effects were found against HPV31, 33, 45, 52 in the vaccinated group. For HPV 6, 59 and 66 no significant reductions in numbers of incident and persistent infections were found, however (borderline) VL reductions following vaccination were

**Competing interests:** C.J.L.M. has received speakers' fee from SPMSD/Merck and has served occasionally on the scientific advisory board (expert meeting) of Qiagen, SPMSD/Merck and GSK. He has been co-investigator on a SPMSD sponsored HPV vaccination trial in males, for which his institute has received funding. He is part-time director and minority stock holder of Self-Screen b.v., a spin off company of VUMC, and has a very small number of Qiagen shares. Until April 2016 he had minority stock of Diassay b.v. This does not alter our adherence to PLOS ONE policies on sharing data and materials.

observed for HPV6 ( $p = 0.01$ ), 59 ( $p = 0.10$ ) and 66 ( $p = 0.03$ ), suggesting a minor effect of the vaccine on the VL level of these HPV types. Overall, vaccination resulted in infections with slightly lower VL, irrespective of HPV type.

## Conclusions

In conclusion, vaccination with the bivalent HPV16/18 vaccine results in significantly reduced numbers of HPV16 and 18 incidence rates and reduced VL in breakthrough infections. Significant reductions in incident and/or persistent HPV31, 33, 35 and 45 infections were found, but no significant effect was observed on the VL for infections with these types. For the other non-vaccine HPV types no reduction in incident and/or persistent infections were found, but overall the VL tended to be somewhat lower in vaccinated women.

## Introduction

Cervical intraepithelial lesions (CIN) and cervical cancer are caused by persistent high-risk human papillomavirus (hrHPV) infections [1]. Progression from initial infection to precancerous lesions and ultimately cervical cancer can take decades [2, 3]. CIN2 and CIN3 are the most advanced precursor lesions for cervical cancer and are accepted by the WHO as surrogate cervical cancer endpoint markers for vaccine efficacy. Later, persistent HPV infections (>6 months) were also accepted as adequate surrogate endpoint markers [4]. Among hrHPV types, HPV16 and 18 cause approximately 70% of all cervical cancers worldwide. A further 20% of the worldwide cervical cancer burden is added by HPV31, 33, 45, 52 and 58 [5].

To prevent HPV-associated malignancies, three highly efficacious prophylactic recombinant vaccines are currently registered and commercially available. The bivalent vaccine protects against HPV16 and 18, quadrivalent adds HPV6 and 11, and nonavalent further adds HPV31, 33, 45, 52 and 58 [6]. Varying degrees of cross-protection have been described against non-vaccine oncogenic HPV types for the bivalent and the quadrivalent vaccines [7–15]. The bivalent vaccine does seem to generate a broader cross-protective response against non-vaccine HPV types associated with CIN2+ (HPV31, 33, 39, 45 and 51) than the quadrivalent vaccine (limited to HPV31) [16]. Interestingly, limited VE has also been reported for the bivalent vaccine against HPV6 and 11 infections [17], resulting in a reduced occurrence of anogenital warts in England [18, 19]. However, these results should be further confirmed. Cross-protection is currently a heavy focus of investigation in the field and cross-protective effects are being (partly) attributed to the AS04 adjuvant (3-*O*-desacyl-4'-monophosphoryl lipid A adsorbed on aluminum salt), which appears to induce enhanced and longer-lasting antibody titers than the aluminum hydroxyphosphate sulfate adjuvant used in the quadri- and nonavalent vaccines [20]. Vaccination with the bivalent HPV vaccine also offers high, but not complete, protection against vaccine type incident infections [21, 22].

The protective effect against persistent HPV16/18 and some non-vaccine type HPV infections and associated CIN2+ lesions [11, 12, 16, 21–24], combined with the lower VE against incident HPV16/18 infections [21, 22], suggest that infections that establish in vaccinated individuals are very unlikely to persist. It has been previously described that persistence of HPV infection is associated with high viral load (VL) [25, 26]. Lower VL of these HPV types could be expected among HPV infections in vaccinated compared to non-vaccinated individuals. Consequently, longitudinal VL measurements might give additional insights in vaccine effects.

In 2009, bivalent HPV vaccination was introduced in the Netherlands. At the same time, the HPV Amongst Vaccinated and Non-vaccinated Adolescents (HAVANA) cohort study was started to monitor vaccine effectiveness [12, 27, 28]. Recently, detailed analyses of this cohort were published, including type-specific and pooled vaccine effectiveness estimates against incident and persistent infections. A high vaccine effectiveness against HPV16 and HPV18 infections was shown, as well as significant cross-protection against HPV31, 33 and 45 [12]. The present analysis aimed to use the same cohort study to assess type-specific (TS) incident and persistent HPV infections and to compare their VLs in both vaccinated and non-vaccinated women in order to see whether TS VL measurements could be used as a marker for HPV type protection.

## Materials and methods

### Study population and HPV DNA genotyping

Vaginal self-samples were obtained annually from participants of the “*HPV Amongst Vaccinated and Non-vaccinated Adolescents*” (HAVANA) observational cohort study, which has been described previously [28]. In short, 29,162 girls, aged 14–16 years old, eligible for catch-up vaccination, were invited for participation in 2009 and 2010. From the invitees, 6% consented to study participation. The inclusion criteria for the present analysis were girls having received either three doses of the bivalent HPV vaccine, or zero doses. Study characteristics for the present population were compared to a previous analysis of this study cohort [12]. Informed consent was obtained from study participants and both parents (if possible), or a legal representative. This study was approved by the Medical Ethics Committee of VUmc Amsterdam (2009/22). For the present analysis, a baseline and seven follow-up samples were available per study participant.

Total DNA was isolated from 200µl of self-samples using the MagNAPure96 platform (Roche Diagnostics) and eluted in 100µl elution buffer. A phocine herpesvirus-1 spike was added to each sample as an internal control for DNA isolation. Isolated DNA was genotyped using the analytically sensitive SPF10-DEIA-LiPA<sub>25</sub> platform [29–31].

### Plasmid transformation, isolation and quantification

Ampicillin resistant plasmids for HPV6, 11, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 66 were kindly provided by the HPV Reference Center, Karolinska Institutet, Sweden. Plasmids were transformed into heat-competent NEB 5-alpha *E. coli* (High Efficiency) (New England Biolabs) according to the manufacturer’s protocol and the cell suspension was subsequently plated on MH agar plates containing ampicillin. Plates were incubated overnight at 37°C and single colonies were transferred to liquid LB medium containing ampicillin. Cultures were grown while shaking to OD<sub>600</sub> = 2–3, and then centrifuged at 5000g for ten minutes. Plasmid was isolated from cell pellets using GeneJet Plasmid Midiprep kit (Thermo Scientific) according to the manufacturer’s protocol. Isolated plasmid was eluted in 40µl elution buffer and quantitated in triplicate in three individual experiments to minimize assay variation using PicoGreen dsDNA quantification assay (ThermoFisher).

### Assay performance and viral load quantification

New quantitative VL assays for HPV6, 11, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 66 were developed in line with HPV16/18 assays described previously [26]. Primer and probe sequences are shown in [S1 Table](#). Quantifications were performed on the LightCycler480 (Roche Diagnostics) platform. Calibration curves were generated for individual assays using

triple measurements of twofold dilution series as described previously [26]. Detection limits were set at the lowest concentration detectable in the linear spectrum of the calibration curve (S1 Table). Assay specificity was determined by testing TS reagents with different plasmids at high concentrations (> 1x10<sup>7</sup> genome equivalents (GEq)/reaction). To assess possible over- and underscoring of assays, nine dilutions from each calibration curve were tested in the presence and absence of high concentrations (>1.7x10<sup>5</sup> GEq/reaction) of the eight most prevalent hrHPV types in this study; HPV16, 18, 31, 33, 45, 51, 52 and 58. All measurements were performed in singleplex for optimal sensitivity and normalized by measuring cellular content via beta-actin qPCR [32]. Samples TS HPV DNA positive by genotyping, but without measurable VL were assigned an arbitrary VL of 10<sup>-5</sup> copies/cell [33].

### Infection and incidence rate definitions

To be included in the analysis of any HPV type, study participants had to be genotyping negative at baseline for that specific HPV type. HPV (baseline) positivity was assessed on a per type basis. Study participants could be included independently in multiple TS analyses. An exclusion for any one HPV type did not result in *de facto* exclusion for other HPV types.

Infection definitions are explained in Table 1. Any TS HPV measurement following a TS HPV negative baseline was considered an incident infection. Clearing infections were defined as a single HPV TS positive measurement with no new HPV TS positive measurement identified within two years (Table 1C–1E). Infection persistence was defined as at least two subsequent HPV TS positive measurements (±12 months between samples; Table 1G–1J). If a study participant fulfilled both the criteria for a clearing and a persistent infection for a single HPV type, only the first infection was included (Table 1F and 1K). For VL analysis of persistent infections, the first positive samples were quantified, since load at first detection is a major determinant of HPV persistence [34].

Incidence rates (IR) were calculated for incident clearing (IRI) and persistent (IRP) infections to compare with VL measurements. Person-time for any infection was only counted when at least two subsequent measurements were available after the first HPV TS positive sample. As the exact moment of infection could not be determined, interval censoring was applied for incident clearing infections after the first TS HPV positive measurement. For persistent infections one more sample after the initial TS HPV positive sample was included after which the participant was censored. IRI and IRP were adjusted for factors associated with vaccination status, as reported previously: age, urbanization degree, ever smoked, ever used contraception,

Table 1. Infection definitions as used for this study.

	Baseline TS HPV	Per round (annual) TS HPV					Action taken
A	+						Excluded
B	-	-	-	-	-	-	HPV negative
C	-	+	-	-	-	-	Incident clearing
D	-	+	-	-	+	-	Incident clearing
E	-	+	-	0	0	0	Incident clearing
F	-	+	-	-	+	+	Incident clearing
G	-	+	-	+	-	+	Excluded
H	-	+	+	-	-	-	Incident persistent
I	-	+	+	-	+	+	Incident persistent
J	-	+	+	+	+	+	Incident persistent
K	-	+	+	-	-	+	Incident persistent
L	-	+	+	0	0	0	Incident persistent

<https://doi.org/10.1371/journal.pone.0212927.t001>

ever had sex [12]. Adjusted (a)IRI and aIRP ratios comparing vaccinated and non-vaccinated participants were calculated with 95% confidence intervals (CI) using a Poisson model. Ratios not overlapping unity were considered statistically significant.

Participants supplied samples annually. Participants were excluded from a type-specific (TS) analysis if positive at baseline for that human papillomavirus (HPV) type (A). Following a negative TS HPV baseline measurement, participants were either negative, incident clearing or incident persistent for that HPV type (B-F, H-L). Intermittent infections (G) were excluded due to uncertainty about true incidence or latent persistence. Infections meeting definitions for both clearing and persistent infections were only included based on the first infection identified for that HPV type (F, K).

### Statistical analysis

Statistical analysis was performed using GraphPad Prism 7 (GraphPad Software) and SAS 9.4 (SAS Institute Inc.). Two-tailed p-values <0.05 were considered statistically significant. Differences in study populations for the present analysis and the study population as published by Donken *et al.* [12] were assessed via chi square and student's t-test. VL differences were initially assessed using the Mann-Whitney U test based on vaccination status for the complete dataset and then stratified for incident and persistent infections.

## Results

### Study characteristics

From a total of 1832 HAVANA study participants, 115 women were excluded due to a missing sample at baseline or incomplete or unknown vaccination status. 1717 non-vaccinated (n = 769; 44.8%) or three-dose vaccinated (n = 948; 55.2%) girls were included in this analysis. Characteristics of the selected study population are listed in Table 2 and did not differ significantly for any of the tested variables when compared to the study population described by Donken *et al.* [12]. In total, 621 incident clearing infections (327 in vaccinated and 294 in non-

Table 2. General characteristics at baseline for the study population subset.

	<i>Present analysis</i>	<i>Donken et al. [12]</i>	
<b>Vaccination coverage</b>			
n (%)	948 (55)	875 (54)	p = 0.32
<b>Mean age (range)</b>			
Non-vaccinated	15 (14–17)	15 (14–17)	p = 0.99
Vaccinated	15 (14–16)	15 (14–16)	p = 0.90
<b>Low urbanisation</b>			
Non-vaccinated	227 (31)	227 (31)	p = 0.95
Vaccinated	126 (14)	114 (13)	
<b>Ever smoked</b>			
Non-vaccinated	249 (38)	248 (38)	p = 0.89
Vaccinated	282 (32)	267 (32)	
<b>Ever used contraception</b>			
Non-vaccinated	314 (42)	314 (42)	p = 0.82
Vaccinated	350 (38)	332 (38)	
<b>Ever had sex</b>			
Non-vaccinated	210 (28)	210 (28)	p = 0.97
Vaccinated	201 (22)	187 (22)	

<https://doi.org/10.1371/journal.pone.0212927.t002>

**Table 3. Infection characteristics of the study cohort, stratified by vaccination status and HPV type for incident clearing infections.**

	Incident clearing infections									
	Non-vaccinated				Vaccinated				IRI Ratio	
	Cases	PY	IR per 1000 PY	Adjusted	Cases	PY	IR per 1000 PY	Adjusted	Ratio (95%CI)	Adjusted
HPV6	35	3490	10.03 (7.20–13.97)	9.04 (6.20–13.17)	39	4153	9.39 (6.86–12.85)	8.24 (5.82–11.67)	0.94 (0.59–1.48)	0.91 (0.55–1.50)
HPV11	10	3625	2.76 (1.48–5.13)	2.33 (1.13–4.82)	12	4266	2.81 (1.60–4.95)	2.79 (1.53–5.08)	1.02 (0.44–2.36)	1.20 (0.47–3.02)
HPV16	22	3487	6.31 (4.15–9.58)	5.53 (3.37–9.05)	9	4279	2.10 (1.10–4.04)	2.12 (1.08–4.14)	0.33 (0.15–0.72)	<b>0.38 (0.17–0.87)</b>
HPV18	12	3567	3.37 (1.91–5.93)	1.52 (0.67–3.48)	13	4270	3.05 (1.77–5.24)	1.41 (0.64–3.08)	0.91 (0.41–1.98)	0.92 (0.39–2.21)
HPV31	22	3544	6.21 (4.09–9.43)	5.36 (3.24–8.89)	7	4285	1.63 (0.78–3.43)	1.70 (0.80–3.59)	0.26 (0.11–0.62)	<b>0.32 (0.13–0.76)</b>
HPV33	15	3604	4.16 (2.51–6.91)	3.62 (1.96–6.67)	5	4284	1.17 (0.49–2.80)	1.12 (0.45–2.77)	0.28 (0.10–0.77)	<b>0.31 (0.11–0.88)</b>
HPV35	5	3642	1.37 (0.57–3.30)	0.60 (0.11–3.20)	0	4317	-	-	-	-
HPV39	24	3544	6.78 (4.54–10.10)	5.12 (3.12–8.39)	21	4190	5.01 (3.27–7.69)	3.82 (2.31–6.31)	0.74 (0.41–1.33)	0.75 (0.39–1.41)
HPV45	13	3610	3.60 (2.09–6.20)	2.75 (1.33–5.67)	1	4320	0.23 (0.03–1.64)	0.20 (0.03–1.47)	0.06 (0.01–0.49)	<b>0.07 (0.01–0.56)</b>
HPV51	46	3343	13.76 (10.31–18.37)	10.26 (7.25–14.59)	62	3917	15.83 (12.34–20.30)	14.56 (11.07–19.15)	1.15 (0.79–1.68)	1.42 (0.93–2.16)
HPV52	29	3468	8.37 (5.81–12.03)	7.19 (4.71–10.99)	22	4151	5.30 (3.49–8.05)	4.85 (3.09–7.61)	0.63 (0.36–1.10)	0.67 (0.38–1.21)
HPV56	30	3502	8.57 (5.99–12.25)	6.85 (4.47–10.52)	36	4160	8.66 (6.24–12.00)	8.46 (5.98–11.96)	1.01 (0.62–1.64)	1.23 (0.72–2.12)
HPV58	9	3623	2.48 (1.29–4.77)	1.94 (0.88–4.30)	7	4276	1.64 (0.78–3.43)	1.36 (0.60–3.12)	0.66 (0.25–1.77)	0.70 (0.25–1.95)
HPV59	15	3627	4.13 (2.49–6.86)	3.56 (1.98–6.38)	17	4234	4.02 (2.50–6.46)	3.36 (1.96–5.78)	0.97 (0.48–1.94)	0.95 (.45–1.97)
HPV66	40	3466	11.54 (8.47–15.73)	10.97 (7.78–15.46)	43	4028	10.68 (7.72–14.39)	9.22 (6.60–12.87)	0.93 (0.60–1.42)	0.84 (0.53–1.34)

Observed person years (PY) are shown, with incidence rates (IR) for incident (IRI) and persistent (IRP) infections. The ratio represents vaccinated IR per 1000PY divided by non-vaccinated IR per 1000PY. Rates were adjusted for age, urbanization degree, ever smoked, ever used contraception and ever had sex according to [12]. Significant results are displayed in bold.

-: No value could be calculated due to zero case incidence.

#: Model did not converge due to insufficient data points.

<https://doi.org/10.1371/journal.pone.0212927.t003>

vaccinated) and 484 incident persistent infections (232 in vaccinated and 252 in non-vaccinated) were identified. The most prevalent HPV types by absolute count in both vaccine and non-vaccine recipients were HPV51 (n = 123; n = 100 respectively), HPV66 (n = 88; n = 65), and HPV52 (n = 59; n = 57), as shown in Tables 3 and 4.

The present analysis was compared to a previous epidemiological description of this cohort study [12] and differences were assessed.

### HPV16/18 vaccination leads to reduced incidence of HPV16/18, with some cross-protective effects

Incidence rates and ratios were calculated per thousand observed person years for vaccinated and non-vaccinated individuals and adjusted according to Donken *et al.* [12], as shown in Tables 3 and 4. In line with results reported by Donken *et al.* [12], we find significantly less incident clearing and persistent HPV16 infections in the vaccinated group (aIRI ratio: 0.38; 95%CI: 0.17–0.87; aIRP ratio: 0.08; 95%CI: 0.02–0.27). For HPV18 no significant effect was observed against clearing infections, but persistent infections did not occur at all in vaccinated individuals (no ratio calculated, vaccinated IRP of zero).

For non-vaccine types, a reduced incidence of incident clearing infections was found in the vaccinated group for HPV31 (aIRI ratio: 0.32, 95%CI: 0.13–0.76), HPV33 (aIRI ratio: 0.31, 95%CI: 0.11–0.88), HPV35 (no ratio calculated, vaccinated aIRI of zero) and HPV45 (aIRI ratio: 0.07, 95%CI: 0.01–0.56). Additionally, less HPV31 persistent infections were found among vaccinated study participants (aIRP ratio: 0.18, 95%CI: 0.06–0.52). Interestingly, more

Table 4. Infection characteristics of the study cohort, stratified by vaccination status and HPV type for incident persistent infections.

	Incident persistent infections									
	Non-vaccinated				Vaccinated				IRP Ratio	
	Cases	PY	IR per 1000 PY	Adjusted	Cases	PY	IR per 1000 PY	Adjusted	Ratio (95%CI)	Adjusted
HPV6	14	3569	3.92 (2.32–6.62)	3.57 (1.97–6.47)	13	4232	3.07 (1.78–5.29)	2.15 (1.11–4.17)	0.78 (0.37–1.67)	0.60 (0.26–1.40)
HPV11	2	3642	0.55 (0.14–2.20)	#	5	4296	1.16 (0.48–2.80)	0.29 (0.04–2.00)	2.12 (0.41–10.92)	#
HPV16	33	3588	9.21 (6.54–12.95)	7.70 (5.02–11.82)	3	4291	0.69 (0.23–2.17)	0.62 (0.20–1.97)	0.08 (0.02–0.25)	<b>0.08 (0.02–0.27)</b>
HPV18	15	3612	4.15 (2.50–6.89)	3.37 (1.76–6.46)	0	4278	-	-	-	-
HPV31	21	3620	5.80 (3.78–8.90)	5.38 (3.25–8.88)	5	4306	1.16 (0.48–2.79)	0.94 (0.35–2.55)	0.20 (0.08–0.53)	<b>0.18 (0.06–0.52)</b>
HPV33	5	3642	1.37 (0.57–3.30)	0.86 (0.27–2.74)	5	4308	1.16 (0.48–2.79)	0.96 (0.35–2.62)	0.85 (0.24–2.92)	1.12 (0.26–4.40)
HPV35	4	3664	1.09 (0.41–2.91)	0.55 (0.13–2.29)	3	4324	0.69 (0.22–2.15)	0.57 (0.16–2.09)	0.64 (0.14–2.84)	1.05 (0.20–5.57)
HPV39	17	3611	4.71 (2.93–7.57)	3.77 (2.12–6.71)	17	4265	3.99 (2.48–6.41)	3.55 (2.09–6.01)	0.85 (0.43–1.66)	0.94 (0.43–2.04)
HPV45	3	3643	0.82 (0.27–2.55)	0.00 (0.00–0.02)	1	4324	0.23 (0.03–1.64)	0.00 (0.00–0.01)	0.28 (0.03–2.70)	0.24 (0.03–2.35)
HPV51	54	3508	15.39 (11.79–20.10)	14.78 (11.03–19.80)	61	4111	14.84 (11.55–19.07)	14.12 (10.79–18.48)	0.96 (0.67–1.39)	0.96 (0.65–1.41)
HPV52	29	3558	8.15 (5.66–11.73)	7.12 (4.68–10.81)	37	4251	8.70 (6.31–12.01)	7.87 (5.52–11.22)	1.07 (0.66–1.74)	1.11 (0.65–1.90)
HPV56	24	3595	6.68 (4.48–9.96)	5.02 (3.06–8.23)	19	4255	4.47 (2.85–7.00)	3.65 (2.20–6.07)	0.67 (0.37–1.22)	0.73 (0.38–1.40)
HPV58	5	3650	1.37 (0.57–3.29)	0.52 (0.13–1.99)	7	4306	1.62 (0.78–3.41)	0.66 (0.20–2.20)	1.19 (0.38–3.74)	1.28 (0.37–4.44)
HPV59	2	3658	0.55 (0.14–2.19)	0.25 (0.03–1.86)	11	4276	2.57 (1.43–4.65)	2.12 (0.10–4.37)	4.71 (1.04–21.23)	<b>8.57 (1.06–69.04)</b>
HPV66	25	3582	6.98 (4.72–10.33)	6.82 (4.46–10.41)	45	4173	10.78 (8.05–14.44)	10.00 (7.26–13.77)	1.55 (0.95–2.52)	1.47 (0.87–2.48)

Observed person years (PY) are shown, with incidence rates (IR) for incident (IRI) and persistent (IRP) infections. The ratio represents vaccinated IR per 1000PY divided by non-vaccinated IR per 1000PY. Rates were adjusted for age, urbanization degree, ever smoked, ever used contraception and ever had sex according to [12]. Significant results are displayed in bold.

-: No value could be calculated due to zero case incidence.

#: Model did not converge due to insufficient data points.

<https://doi.org/10.1371/journal.pone.0212927.t004>

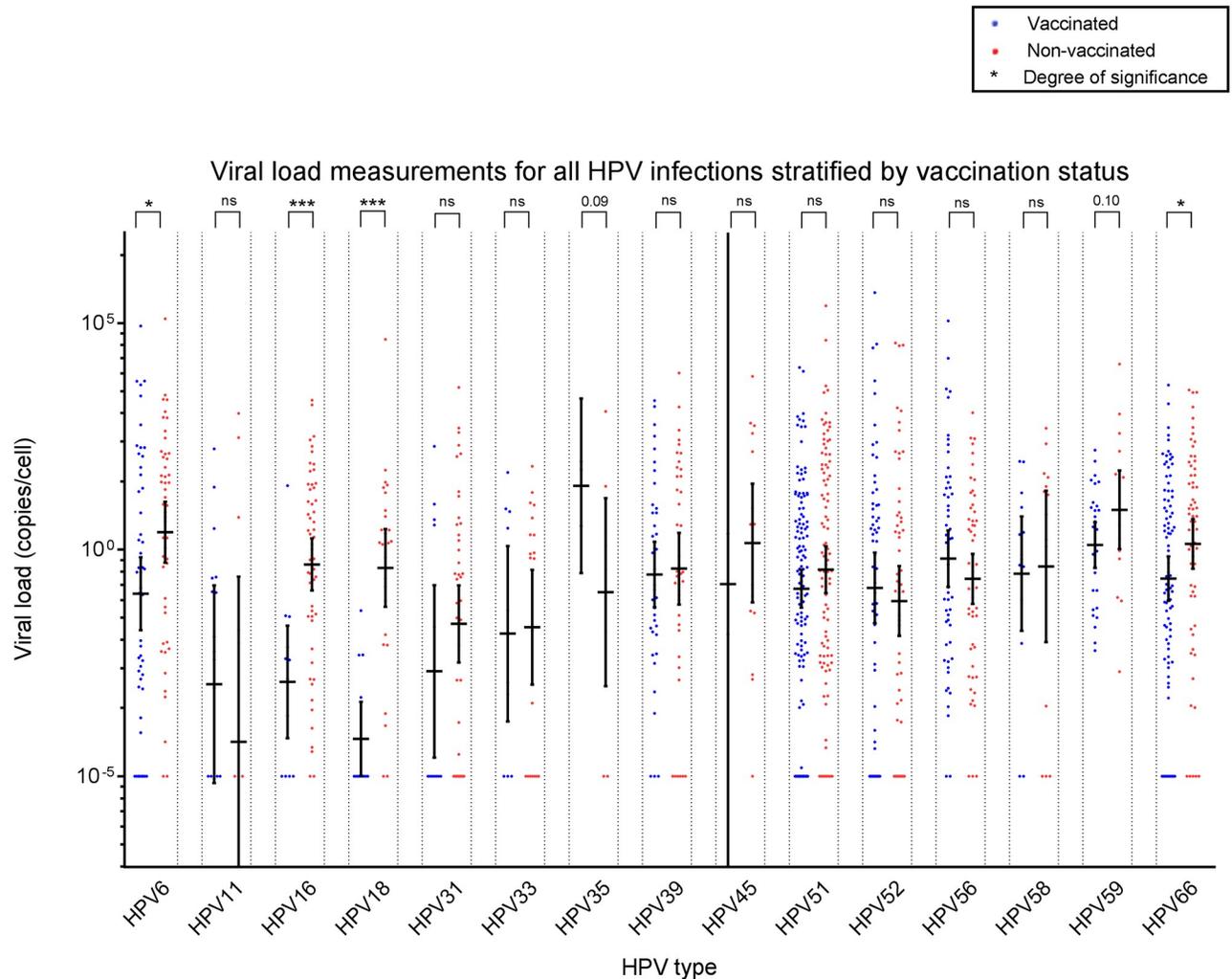
HPV59 persistent infections were found in the vaccinated group (aIRP ratio: 8.57, 95%CI: 1.06–69.04). For other HPV types, no (significant) differences were observed.

### Type-specific quantification of HPV viral load

HPV6, 11, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 66 TS VL assays were specific in the presence of at least 10<sup>7</sup> copies of different HPV types. Sensitivity was <5 GEq/reaction for each individual assay. Reproducibility of VL measurements was assessed by measuring duplicates of a subset of HPV16 (n = 62) and HPV18 (n = 22) samples. Duplicates were found to be on average <0.1 ct values apart (median 0.03; minimum -2.39; maximum 1.66). VL measurements were performed based on genotyping results. Figs 1–3 show scatter plots of VL measurements for study participants in all infections, the clearing infections subset, and persistent infections subset respectively.

### Reduced viral load in breakthrough HPV16/18 infections following HPV16/18 vaccination

VL measurements correlated well with observed reductions in incident/persistent HPV16/18 infections. Median HPV16 VL was significantly lower in vaccine recipients for the total study analysis (p<0.01, Fig 1), as well as stratified analyses for incident clearing (p = 0.02, Fig 2) and persistent infections (p = 0.05, Fig 3). For HPV18, vaccine recipients showed significantly lower median VL in the total analysis (p<0.01, Fig 1) and in the incident clearing analysis (p<0.01, Fig 2). Since no persistent HPV18 infections were identified among vaccinees, no VL comparison could be performed.

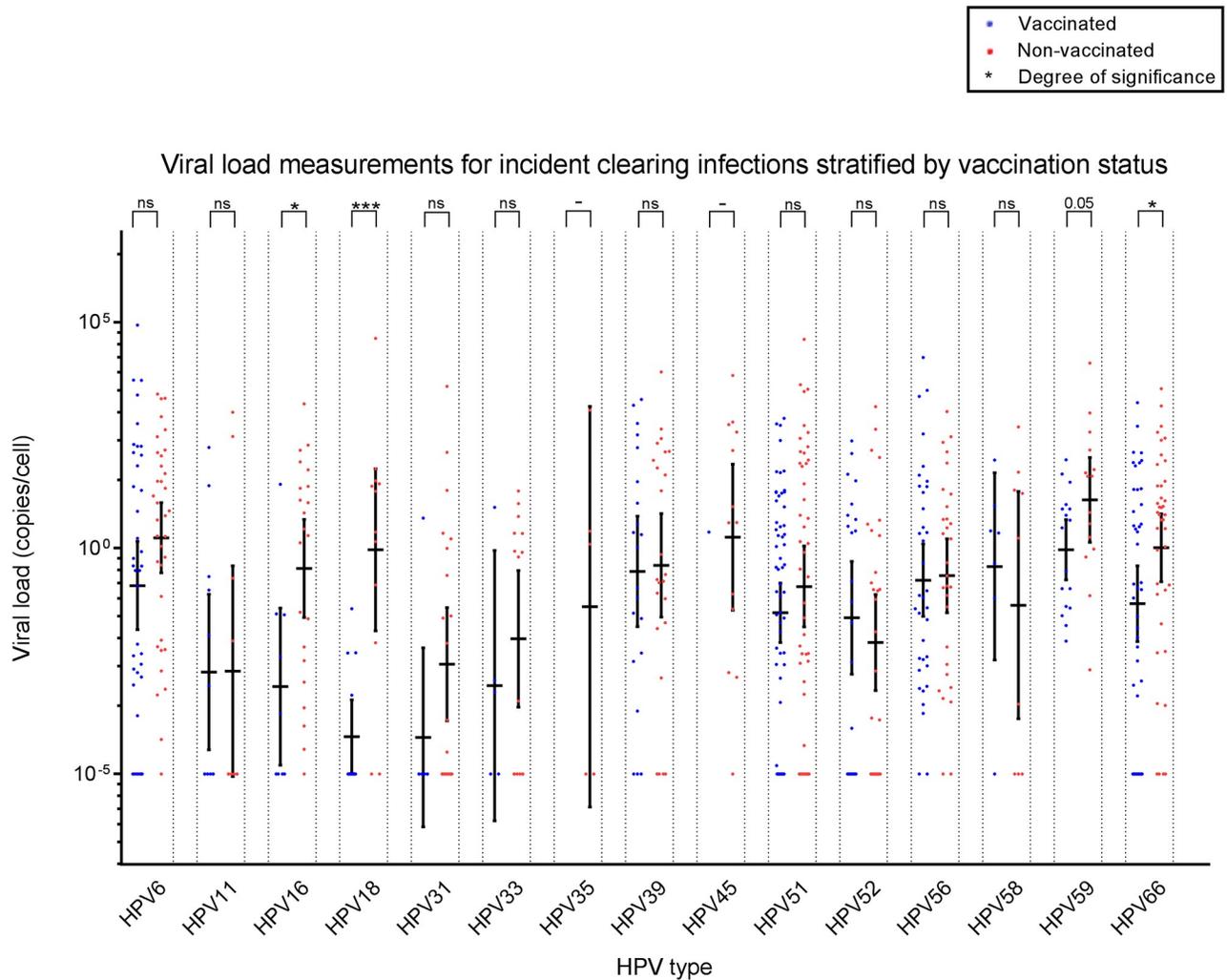


**Fig 1. Viral load measurements of all infections included in this study, expressed in copies per cell on a log<sub>10</sub> scale.** Individual measurements are displayed as blue and red dots for vaccinated and non-vaccinated study participants respectively. For each HPV type median VL values were compared between vaccinated and non-vaccinated individuals. Statistical significance is shown above each HPV type, with \* meaning  $p < 0.05$ ; \*\* meaning  $p < 0.01$ ; \*\*\* meaning  $p < 0.001$ .

<https://doi.org/10.1371/journal.pone.0212927.g001>

### Cross-protective effects on viral load following vaccination are unclear

Despite finding some cross-protection of the bivalent vaccine against HPV31, 33, 35 and 45 (Tables 3 and 4), no significant differences in median VL were observed between groups in any of the analyses for these types. Except for a marginally increased median HPV35 VL in the vaccinated group for the total analysis ( $p = 0.09$ ), although this was based on a limited number of infections ( $n = 3$  vaccinated,  $n = 9$  non-vaccinated). For HPV6, significantly lower median VL values were found in the vaccinated group for the total ( $p = 0.01$ ) and the persistent infections analyses ( $p = 0.05$ ). For HPV59 and 66 (borderline) lower median VL values were found in the vaccinated group for the total ( $p = 0.10$ ;  $p = 0.03$ ) and incident clearing ( $p = 0.05$ ;  $p = 0.04$ ) infection analyses. For HPV58 a marginally lower median VL was observed in vaccinated individuals ( $p = 0.07$ ). For HPV56 an increased median VL was observed in the vaccinated group ( $p = 0.03$ ). The VL results for HPV6, 56, 58, 59 and 66 did not lead to a measurable decrease in the rate of incident or persistent infections in the vaccinated group.



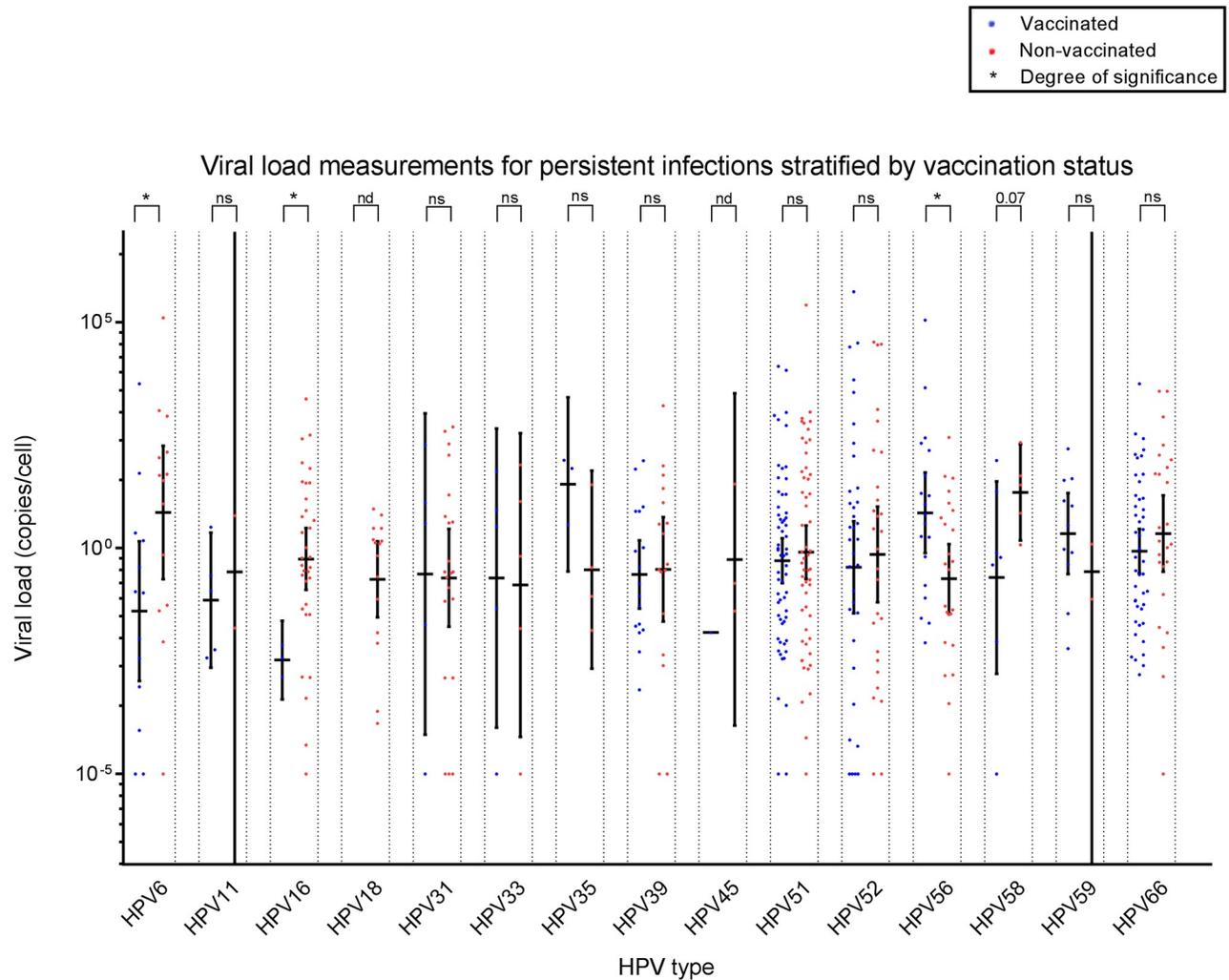
**Fig 2. Viral load measurements in incident infections, expressed in copies per cell on a log10 scale.** Individual measurements are displayed as blue and red dots for vaccinated and non-vaccinated study participants respectively. For each HPV type median VL values were compared between vaccinated and non-vaccinated individuals. Statistical significance is shown above each HPV type, with \* meaning  $p < 0.05$ ; \*\* meaning  $p < 0.01$ ; \*\*\* meaning  $p < 0.001$ .

<https://doi.org/10.1371/journal.pone.0212927.g002>

Interestingly, the median VL of any HPV type in the vaccinated group shows a trend in being slightly lower than in the non-vaccinated group (Figs 1–3). To indicate compatibility between incidence and VL analyses, Table 5 shows summarized results per HPV type, which shows good concordance between analyses for vaccine types, but poor concordance for non-vaccine types.

### Discussion

In this study, newly developed and existing assays [26] were used to measure VL of HPV6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 66 infections on a prospective longitudinal observational cohort study of young women who were eligible for HPV vaccination according to a three-dose schedule. To our knowledge, we are the first to study effects of HPV vaccination on the VL of HPV infections. We confirm that vaccination with the bivalent vaccine leads to significant reductions in the incidence of clearing and/or persistence of HPV16/18



**Fig 3. Viral load measurements in persistent infections, expressed in copies per cell on a log<sub>10</sub> scale.** Individual measurements are displayed as blue and red dots for vaccinated and non-vaccinated study participants respectively. For each HPV type median VL values were compared between vaccinated and non-vaccinated individuals. Statistical significance is shown above each HPV type, with \* meaning  $p < 0.05$ ; \*\* meaning  $p < 0.01$ ; \*\*\* meaning  $p < 0.001$ .

<https://doi.org/10.1371/journal.pone.0212927.g003>

infections, in line with previous results for a different sample subset of this study by Donken *et al.* [12]. In addition, we show that vaccination is associated with significant reductions in median VL of breakthrough incident clearing and persistent HPV16/18 infections. Vaccination with the bivalent vaccine also induced a reduction in both incident clearing and persistent HPV31 infections as well as reductions in incident clearing HPV33 and 45 infections. However, these findings were not associated with any reductions in VL of these HPV types following vaccination. Our findings suggest that vaccination is significantly associated with reduction in VL in breakthrough vaccine type infections, possibly by limiting the capacity of the virus to cause a persistent infection. However, despite confirming cross-protection against HPV31, 33 and 45, the effect on VL is not significantly altered, suggesting that cross-protection against non-vaccine HPV types is either complete (preventing infections in a similar fashion to vaccine types) or absent (not measurable by VL in this study).

Table 5. Summary and compatibility of HPV infection incidence with viral load.

HPV type	Vaccinated compared to non-vaccinated groups; effects measured on:				
	Infection incidence rate		Viral load		
	Incident clearing	Incident persistent	Total infections	Clearing infections	Persistent infections
6	-	-	Reduced	-	Reduced
11	-	-	-	-	-
16	Reduced	Reduced	Reduced	Reduced	Reduced
18	-	Reduced <sup>1</sup>	Reduced	Reduced	- <sup>1</sup>
31	Reduced	Reduced	-	-	-
33	Reduced	-	-	-	-
35	Reduced <sup>1</sup>	-	Increased <sup>2</sup>	- <sup>1</sup>	-
39	-	-	Reduced <sup>2</sup>	-	-
45	Reduced	-	-	- <sup>1</sup>	-
51	-	-	-	-	-
52	-	-	-	-	-
56	-	-	-	-	Increased
58	-	-	-	-	Reduced <sup>2</sup>
59	-	Increased	Reduced <sup>2</sup>	Reduced	-
66	-	-	Reduced	Reduced	-

Reduced/increased incidence or viral load implies a reduction in the vaccinated group compared to the non-vaccinated group.

<sup>1</sup>: Calculation for p-value could not be performed due to complete absence of infections in the vaccinated group.

<sup>2</sup>: Borderline significant results (0.05 < p < 0.1).

<https://doi.org/10.1371/journal.pone.0212927.t005>

Cross-protection has been previously described for HPV31, 33, 35, 45, 52 and 58 [7–10]. We could confirm some of these effects in this study (HPV31, 33, 35 and 45), but no significant VL reductions in the vaccinated group were found for either type, despite a reduced effect, although HPV33 and 45 infections are uncommon in this study (n = 10 for HPV33, n = 4 for HPV45). For HPV6, 59 and 66 (borderline) significant median VL reductions were found among vaccine recipients, although this did not translate into any measurable reduction of incident clearing or persistent infections for these HPV types in this study. Cross-protection is less likely against HPV6 and 66, as they belong to a different HPV species than HPV16 or 18 [35]. For HPV59, despite it being closely related to HPV18, the SPF10-DEIA-LiPA<sub>25</sub> genotyping assay has a relatively poor detection sensitivity [36], which might explain the lack of concordance between HPV59 VL and incidence results.

Vaccination (with any of the currently available virus-like particle (VLP) based vaccines) primarily leads to sterilizing immunity against HPV16/18 via type-restricted neutralizing antibodies, preventing the majority of incident and persistent infections [37]. We report significantly lower median VL values in vaccinated individuals with HPV16/18 breakthrough infections. Our results could suggest that the immune response protecting against HPV infection acquisition could be different from the immune response responsible for viral control. This effect might be facilitated by a T cell mediated immune response limiting viral replication, which is the proposed mechanism leading to cross-protection against non-vaccine type associated CIN2+ [37, 38], and which could help explain results described by Harper *et al.* [16]. This could result in infections with lower VL levels, which have been associated with clearance of infection [39, 40], while high HPV16/18 VL levels have been associated with persisting infections [25, 26].

While we are unable to clearly associate vaccine effects on incidence of infections with VL, we do observe a general trend where median VL for any HPV type was lower for vaccine

recipients than non-vaccinated individuals. If these findings are indeed caused by the vaccine, they might help alleviate or prevent long-term disease effects by dampening infection development through reduced viral load.

Several limitations need to be addressed for this study. First, we included participants based on baseline TS HPV DNA negative samples, since serology was only available for a subset of our cohort. While baseline DNA positivity is informative for the present sample, it does not inform about any past exposure to HPV and any possible effects on VL. Fortunately, due to low sexual activity at inclusion for this study (Table 2), we believe prior infections should be of limited impact for the present analysis. Second, our categorical definitions of incident clearing and persistent infections (Table 1), though pragmatic and in line with previous literature use, might not accurately represent a natural situation and do not take possible deposition of HPV into account [41]. Incident persistent infections should suffer less from HPV depositions, as repeated detection of deposition events one year apart seems unlikely. Based on a previous study describing TS clearance windows for individual HPV types, one year for persistent infections is adequate in general [42]. Ideally, longer intervals should be observed before infections can be truly considered persistent. However, within this study of young, vaccinated women, infections are relatively scarce and longer interval persistent infections would lead to sharp reductions in incidence, prohibiting statistical interpretation of results. Our definitions also lead to exclusion of intermittent infections. For intermittence, it is unclear whether the observed infection is actually persistent with potential latency in between, or actual a repeated incidence of the same genotype. Since, previous research has shown that relative risk for repeat infections might be different from initial acquisition, these infections were excluded [43]. Third, VL is a highly heterogeneous parameter [26, 44]. Combined with a relatively low number of total infections in this study, this could explain why VL point estimates (Figs 1–3) are often lower in the vaccinated group than in the non-vaccinated group; yet no statistical significance is reached in comparisons and compatibility between incidence rates and VL results is low (Table 5), except for vaccine types HPV16/18. Finally, during VL measurements, a number of samples remained VL negative, despite positive genotyping results. For these samples, an artificial VL was assigned [33]. This method was chosen over assigning a VL at the detection limit of the HPV type tested, since this would lead to exaggerated results when correcting for cellular content.

## Conclusions

Combined, our results suggest that vaccination against HPV16/18 affects VL in breakthrough infections when compared to non-vaccinated women. Although some effects are observed against non-vaccine types, this study is insufficiently powered to suggest a clear correlation between vaccination and effects on VL of non-vaccine HPV types. Further population-based studies are required to identify which effects are truly causal to and maintained by the vaccine.

## Supporting information

**S1 Table. Sequences of primers and probes used for the quantification of different HPV types and limits of detection (LOD) of respective assays.**

(DOCX)

## Acknowledgments

The authors would like to thank Carina Eklund and Joakim Dillner for providing HPV plasmids for the various types analyzed in this study, Hans Bogaards and Maarten Schipper for help with statistical analysis, and Suzan Leussink for technical support.

## Author Contributions

**Conceptualization:** Pascal van der Weele, Robine Donken, Hester de Melker, Chris J. L. M. Meijer, Audrey J. King.

**Formal analysis:** Pascal van der Weele, Robine Donken.

**Funding acquisition:** Audrey J. King.

**Investigation:** Pascal van der Weele, Martijn Breeuwsma, Robine Donken, Elske van Logchem, Naomi van Marm-Wattimena, Audrey J. King.

**Methodology:** Pascal van der Weele, Martijn Breeuwsma.

**Project administration:** Audrey J. King.

**Resources:** Hester de Melker, Audrey J. King.

**Supervision:** Chris J. L. M. Meijer, Audrey J. King.

**Validation:** Pascal van der Weele, Martijn Breeuwsma.

**Writing – original draft:** Pascal van der Weele.

**Writing – review & editing:** Pascal van der Weele, Martijn Breeuwsma, Robine Donken, Elske van Logchem, Naomi van Marm-Wattimena, Hester de Melker, Chris J. L. M. Meijer, Audrey J. King.

## References

1. Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, et al. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *The Journal of pathology*. 1999; 189(1):12–9. [https://doi.org/10.1002/\(SICI\)1096-9896\(199909\)189:1<12::AID-PATH431>3.0.CO;2-F](https://doi.org/10.1002/(SICI)1096-9896(199909)189:1<12::AID-PATH431>3.0.CO;2-F) PMID: 10451482.
2. Vink MA, Bogaards JA, van Kemenade FJ, de Melker HE, Meijer CJ, Berkhof J. Clinical progression of high-grade cervical intraepithelial neoplasia: estimating the time to preclinical cervical cancer from doubly censored national registry data. *American journal of epidemiology*. 2013; 178(7):1161–9. <https://doi.org/10.1093/aje/kwt077> PMID: 23897645.
3. Ho GY, Bierman R, Beardsley L, Chang CJ, Burk RD. Natural history of cervicovaginal papillomavirus infection in young women. *The New England journal of medicine*. 1998; 338(7):423–8. <https://doi.org/10.1056/NEJM199802123380703> PMID: 9459645.
4. IARC HPV Working Group. Primary End-points for Prophylactic HPV Vaccine Trials. Lyon (FR)2014.
5. de Sanjose S, Quint WG, Alemany L, Geraets DT, Klaustermeier JE, Lloveras B, et al. Human papillomavirus genotype attribution in invasive cervical cancer: a retrospective cross-sectional worldwide study. *The Lancet Oncology*. 2010; 11(11):1048–56. [https://doi.org/10.1016/S1470-2045\(10\)70230-8](https://doi.org/10.1016/S1470-2045(10)70230-8) PMID: 20952254.
6. Joura EA, Giuliano AR, Iversen OE, Bouchard C, Mao C, Mehlsen J, et al. A 9-valent HPV vaccine against infection and intraepithelial neoplasia in women. *The New England journal of medicine*. 2015; 372(8):711–23. <https://doi.org/10.1056/NEJMoa1405044> PMID: 25693011.
7. Scherpenisse M, Schepp RM, Mollers M, Meijer CJ, Berbers GA, van der Klis FR. Characteristics of HPV-specific antibody responses induced by infection and vaccination: cross-reactivity, neutralizing activity, avidity and IgG subclasses. *PLoS one*. 2013; 8(9):e74797. <https://doi.org/10.1371/journal.pone.0074797> PMID: 24058629.
8. Malagon T, Drolet M, Boily MC, Franco EL, Jit M, Brisson J, et al. Cross-protective efficacy of two human papillomavirus vaccines: a systematic review and meta-analysis. *The Lancet infectious diseases*. 2012; 12(10):781–9. [https://doi.org/10.1016/S1473-3099\(12\)70187-1](https://doi.org/10.1016/S1473-3099(12)70187-1) PMID: 22920953.
9. Harari A, Chen Z, Rodriguez AC, Hildesheim A, Porras C, Herrero R, et al. Cross-protection of the Bivalent Human Papillomavirus (HPV) Vaccine Against Variants of Genetically Related High-Risk HPV Infections. *The Journal of infectious diseases*. 2016; 213(6):939–47. <https://doi.org/10.1093/infdis/jiv519> PMID: 26518044.
10. Wheeler CM, Castellsague X, Garland SM, Szarewski A, Paavonen J, Naud P, et al. Cross-protective efficacy of HPV-16/18 AS04-adjuvanted vaccine against cervical infection and precancer caused by

- non-vaccine oncogenic HPV types: 4-year end-of-study analysis of the randomised, double-blind PATRICIA trial. *The Lancet Oncology*. 2012; 13(1):100–10. [https://doi.org/10.1016/S1470-2045\(11\)70287-X](https://doi.org/10.1016/S1470-2045(11)70287-X) PMID: 22075170.
11. Pollock KGJ. Sustained cross-protection of the bivalent HPV vaccine. *The Journal of infectious diseases*. 2018; 217(10):1515–6. <https://doi.org/10.1093/infdis/jiy068> PMID: 29408966.
  12. Donken R, King AJ, Bogaards JA, Woestenbergh PJ, Meijer C, de Melker HE. High effectiveness of the bivalent HPV vaccine up to six years post-vaccination against incident and persistent HPV infections in young Dutch females. *The Journal of infectious diseases*. 2018; 217(10):1579–89. <https://doi.org/10.1093/infdis/jiy067> PMID: 29409034.
  13. Woestenbergh PJ, King AJ, van Benthem BHB, Donken R, Leussink S, van der Klis FRM, et al. Bivalent Vaccine Effectiveness Against Type-Specific HPV Positivity: Evidence for Cross-Protection Against Oncogenic Types Among Dutch STI Clinic Visitors. *The Journal of infectious diseases*. 2018; 217(2):213–22. <https://doi.org/10.1093/infdis/jix582> PMID: 29140439.
  14. Brown DR, Kjaer SK, Sigurdsson K, Iversen OE, Hernandez-Avila M, Wheeler CM, et al. The impact of quadrivalent human papillomavirus (HPV; types 6, 11, 16, and 18) L1 virus-like particle vaccine on infection and disease due to oncogenic nonvaccine HPV types in generally HPV-naive women aged 16–26 years. *The Journal of infectious diseases*. 2009; 199(7):926–35. <https://doi.org/10.1086/597307> PMID: 19236279.
  15. Wheeler CM, Kjaer SK, Sigurdsson K, Iversen OE, Hernandez-Avila M, Perez G, et al. The impact of quadrivalent human papillomavirus (HPV; types 6, 11, 16, and 18) L1 virus-like particle vaccine on infection and disease due to oncogenic nonvaccine HPV types in sexually active women aged 16–26 years. *The Journal of infectious diseases*. 2009; 199(7):936–44. <https://doi.org/10.1086/597309> PMID: 19236277.
  16. Harper DM, DeMars LR. HPV vaccines—A review of the first decade. *Gynecologic oncology*. 2017; 146(1):196–204. <https://doi.org/10.1016/j.ygyno.2017.04.004> PMID: 28442134.
  17. Szarewski A, Skinner SR, Garland SM, Romanowski B, Schwarz TF, Apter D, et al. Efficacy of the HPV-16/18 AS04-adjuvanted vaccine against low-risk HPV types (PATRICIA randomized trial): an unexpected observation. *The Journal of infectious diseases*. 2013; 208(9):1391–6. <https://doi.org/10.1093/infdis/jit360> PMID: 24092907.
  18. Howell-Jones R, Soldan K, Wetten S, Mesher D, Williams T, Gill ON, et al. Declining genital Warts in young women in England associated with HPV 16/18 vaccination: an ecological study. *The Journal of infectious diseases*. 2013; 208(9):1397–403. <https://doi.org/10.1093/infdis/jit361> PMID: 24092908.
  19. Canvin M, Sinka K, Hughes G, Mesher D. Decline in genital warts diagnoses among young women and young men since the introduction of the bivalent HPV (16/18) vaccination programme in England: an ecological analysis. *Sexually transmitted infections*. 2016; 93(2):125–8. <https://doi.org/10.1136/sextrans-2016-052626> PMID: 27365492.
  20. Einstein MH, Levin MJ, Chatterjee A, Chakhtoura N, Takacs P, Cateau G, et al. Comparative humoral and cellular immunogenicity and safety of human papillomavirus (HPV)-16/18 AS04-adjuvanted vaccine and HPV-6/11/16/18 vaccine in healthy women aged 18–45 years: follow-up through Month 48 in a Phase III randomized study. *Human vaccines & immunotherapeutics*. 2014; 10(12):3455–65. <https://doi.org/10.4161/hv.36117> PMID: 25483700.
  21. Harper DM. Impact of vaccination with Cervarix (trade mark) on subsequent HPV-16/18 infection and cervical disease in women 15–25 years of age. *Gynecologic oncology*. 2008; 110(3 Suppl 1):S11–7. <https://doi.org/10.1016/j.ygyno.2008.06.029> PMID: 18649932.
  22. Naud PS, Roteli-Martins CM, De Carvalho NS, Teixeira JC, de Borja PC, Sanchez N, et al. Sustained efficacy, immunogenicity, and safety of the HPV-16/18 AS04-adjuvanted vaccine: final analysis of a long-term follow-up study up to 9.4 years post-vaccination. *Human vaccines & immunotherapeutics*. 2014; 10(8):2147–62. <https://doi.org/10.4161/hv.29532> PMID: 25424918.
  23. Apter D, Wheeler CM, Paavonen J, Castellsague X, Garland SM, Skinner SR, et al. Efficacy of human papillomavirus 16 and 18 (HPV-16/18) AS04-adjuvanted vaccine against cervical infection and precancer in young women: final event-driven analysis of the randomized, double-blind PATRICIA trial. *Clinical and vaccine immunology: CVI*. 2015; 22(4):361–73. <https://doi.org/10.1128/CVI.00591-14> PMID: 25651922.
  24. Struyf F, Colau B, Wheeler CM, Naud P, Garland S, Quint W, et al. Post hoc analysis of the PATRICIA randomized trial of the efficacy of human papillomavirus type 16 (HPV-16)/HPV-18 AS04-adjuvanted vaccine against incident and persistent infection with nonvaccine oncogenic HPV types using an alternative multiplex type-specific PCR assay for HPV DNA. *Clinical and vaccine immunology: CVI*. 2015; 22(2):235–44. <https://doi.org/10.1128/CVI.00457-14> PMID: 25540273.
  25. Winer RL, Xi LF, Shen Z, Stern JE, Newman L, Feng Q, et al. Viral load and short-term natural history of type-specific oncogenic human papillomavirus infections in a high-risk cohort of midadult women.

- International journal of cancer Journal international du cancer. 2014; 134(8):1889–98. <https://doi.org/10.1002/ijc.28509> PMID: 24136492.
26. van der Weele P, van Logchem E, Wolffs P, van den Broek I, Feltkamp M, de Melker H, et al. Correlation between viral load, multiplicity of infection, and persistence of HPV16 and HPV18 infection in a Dutch cohort of young women. *Journal of clinical virology: the official publication of the Pan American Society for Clinical Virology*. 2016; 83:6–11. <https://doi.org/10.1016/j.jcv.2016.07.020> PMID: 27522635.
  27. Mollers M, King AJ, Knol MJ, Scherpenisse M, Meijer CJ, van der Klis FR, et al. Effectiveness of human papillomavirus vaccine against incident and persistent infections among young girls: Results from a longitudinal Dutch cohort study. *Vaccine*. 2015; 33(23):2678–83. <https://doi.org/10.1016/j.vaccine.2015.04.016> PMID: 25887090.
  28. Mollers M, Scherpenisse M, van der Klis FR, King AJ, van Rossum TG, van Logchem EM, et al. Prevalence of genital HPV infections and HPV serology in adolescent girls, prior to vaccination. *Cancer Epidemiol*. 2012; 36(6):519–24. <https://doi.org/10.1016/j.canep.2012.07.006> PMID: 22906483.
  29. Kleter B, van Doorn LJ, Schrauwen L, Molijn A, Sastrowijoto S, ter Schegget J, et al. Development and clinical evaluation of a highly sensitive PCR-reverse hybridization line probe assay for detection and identification of anogenital human papillomavirus. *Journal of clinical microbiology*. 1999; 37(8):2508–17. PMID: 10405393.
  30. Kleter B, van Doorn LJ, ter Schegget J, Schrauwen L, van Krimpen K, Burger M, et al. Novel short-fragment PCR assay for highly sensitive broad-spectrum detection of anogenital human papillomaviruses. *The American journal of pathology*. 1998; 153(6):1731–9. [https://doi.org/10.1016/S0002-9440\(10\)65688-X](https://doi.org/10.1016/S0002-9440(10)65688-X) PMID: 9846964.
  31. van Doorn LJ, Molijn A, Kleter B, Quint W, Colau B. Highly effective detection of human papillomavirus 16 and 18 DNA by a testing algorithm combining broad-spectrum and type-specific PCR. *Journal of clinical microbiology*. 2006; 44(9):3292–8. <https://doi.org/10.1128/JCM.00539-06> PMID: 16954263.
  32. Zhao M, Rosenbaum E, Carvalho AL, Koch W, Jiang W, Sidransky D, et al. Feasibility of quantitative PCR-based saliva rinse screening of HPV for head and neck cancer. *International journal of cancer Journal international du cancer*. 2005; 117(4):605–10. <https://doi.org/10.1002/ijc.21216> PMID: 15929076.
  33. Chaturvedi AK, Graubard BI, Pickard RK, Xiao W, Gillison ML. High-risk oral human papillomavirus load in the US population, National Health and Nutrition Examination Survey 2009–2010. *The Journal of infectious diseases*. 2014; 210(3):441–7. <https://doi.org/10.1093/infdis/jiu116> PMID: 24625808.
  34. de Sanjose S, Brotons M, Pavon MA. The natural history of human papillomavirus infection. *Best Pract Res Clin Obstet Gynaecol*. 2018; 47:2–13. <https://doi.org/10.1016/j.bpobgyn.2017.08.015> PMID: 28964706.
  35. de Villiers EM. Cross-roads in the classification of papillomaviruses. *Virology*. 2013; 445(1–2):2–10. <https://doi.org/10.1016/j.virol.2013.04.023> PMID: 23683837.
  36. DDL Diagnostics Laboratory. DNA ELISA kit HPV SPF10, version 1.
  37. Schiller J, Lowy D. Explanations for the high potency of HPV prophylactic vaccines. *Vaccine*. 2018. <https://doi.org/10.1016/j.vaccine.2017.12.079> PMID: 29325819.
  38. Pinto LA, Viscidi R, Harro CD, Kemp TJ, Garcia-Pineros AJ, Trivett M, et al. Cellular immune responses to HPV-18, -31, and -53 in healthy volunteers immunized with recombinant HPV-16 L1 virus-like particles. *Virology*. 2006; 353(2):451–62. <https://doi.org/10.1016/j.virol.2006.06.021> PMID: 16863657.
  39. Carcopino X, Henry M, Mancini J, Giusiano S, Boubli L, Olive D, et al. Two years outcome of women infected with high risk HPV having normal colposcopy following low-grade or equivocal cytological abnormalities: are HPV16 and 18 viral load clinically useful predictive markers? *Journal of medical virology*. 2012; 84(6):964–72. <https://doi.org/10.1002/jmv.23276> PMID: 22499020.
  40. Trevisan A, Schlecht NF, Ramanakumar AV, Villa LL, Franco EL, Ludwig-McGill Study G. Human papillomavirus type 16 viral load measurement as a predictor of infection clearance. *The Journal of general virology*. 2013; 94(Pt 8):1850–7. <https://doi.org/10.1099/vir.0.051722-0> PMID: 23677791.
  41. Malagon T, Burchell AN, El-Zein M, Guenoun J, Tellier PP, Coutlee F, et al. Estimating HPV DNA Deposition Between Sexual Partners Using HPV Concordance, Y Chromosome DNA Detection, and Self-reported Sexual Behaviors. *The Journal of infectious diseases*. 2017; 216(10):1210–8. <https://doi.org/10.1093/infdis/jix477> PMID: 28968731.
  42. Rositch AF, Koshiol J, Hudgens MG, Razzaghi H, Backes DM, Pimenta JM, et al. Patterns of persistent genital human papillomavirus infection among women worldwide: a literature review and meta-analysis. *International journal of cancer Journal international du cancer*. 2013; 133(6):1271–85. <https://doi.org/10.1002/ijc.27828> PMID: 22961444.
  43. Donken R, Knol MJ, Ogilvie GS, Dobson S, King AJ, Singer J, et al. Measuring vaccine effectiveness against persistent HPV infections in observational studies: methodological challenges. *Monitoring the*

HPV vaccination program in the Netherlands: Effects, changing schedule and future perspective 2018. p. 206–22.

44. Snijders PJ, Hogewoning CJ, Hesselink AT, Berkhof J, Voorhorst FJ, Bleeker MC, et al. Determination of viral load thresholds in cervical scrapings to rule out CIN 3 in HPV 16, 18, 31 and 33-positive women with normal cytology. *International journal of cancer Journal international du cancer*. 2006; 119(5):1102–7. <https://doi.org/10.1002/ijc.21956> PMID: 16570279.