



Effects of varying antigens and adjuvant systems on the immunogenicity and safety of investigational tetravalent human oncogenic papillomavirus vaccines: Results from two randomized trials



Pierre Van Damme^{a,*}, Geert Leroux-Roels^b, Philippe Simon^c, Jean-Michel Foidart^{d,1}, Gilbert Donders^{e,2}, Karel Hoppenbrouwers^f, Myron Levin^g, Fabian Tibaldi^h, Sylviane Poncelet^h, Philippe Moris^h, Francis Dessyⁱ, Sandra L. Giannini^h, Dominique Descampsⁱ, Gary Dubin^j

^a Universiteit Antwerpen, Vaccine & Infectious Disease Institute, Centre for the Evaluation of Vaccination, Building R, 2nd Floor, Universiteitsplein 1, 2610 Antwerpen, Belgium

^b Center for Vaccinology, Ghent University and Ghent University Hospital, De Pintelaan 185, 9000 Ghent, Belgium

^c Service de Gynécologie Obstétrique, Hôpital Erasme, Route de Lennik 808, 1070 Bruxelles, Belgium

^d CHR Citadelle, Service de Gynécologie Obstétrique, Boulevard du 12ieme de Ligne 1, 4000 Liège, Belgium

^e Gynaecologie, Heilig Hartziekenhuis, Kliniekstraat 45, 3300 Tienen, Belgium

^f KULeuven, Jeugdgezondheidszorg, Kapucijnenvoer 35/1, 3000 Leuven, Belgium

^g University of Colorado School of Medicine, Building 401, 1784 Racine St., Aurora, CO 80045, USA

^h GlaxoSmithKline Vaccines, Rue de l'Institut 89, 1330 Rixensart, Belgium

ⁱ GlaxoSmithKline Vaccines, Avenue Fleming 20, 1300 Wavre, Belgium

^j GlaxoSmithKline SA, 2301 Renaissance Boulevard, RN0220, King of Prussia, PA 19406, USA

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ABSTRACT

Background: A prophylactic human papillomavirus (HPV) vaccine targeting oncogenic HPV types in addition to HPV-16 and -18 may broaden protection against cervical cancer. Two Phase I/II, randomized, controlled studies were conducted to compare the immunogenicity and safety of investigational tetravalent HPV L1 virus-like particle (VLP) vaccines, containing VLPs from two additional oncogenic genotypes, with the licensed HPV-16/18 AS04-adjuvanted vaccine (control) in healthy 18–25 year-old women.

Methods: In one trial (NCT00231413), subjects received control or one of 6 tetravalent HPV-16/18/31/45 AS04 vaccine formulations at months (M) 0,1,6. In a second trial (NCT00478621), subjects received control or one of 5 tetravalent HPV-16/18/33/58 vaccines formulated with different adjuvant systems (AS04, AS01 or AS02), administered on different schedules (M0,1,6 or M0,3 or M0,6).

Results: One month after the third injection (Month 7), there was a consistent trend for lower anti-HPV-16 and -18 geometric mean antibody titers (GMTs) for tetravalent AS04-adjuvanted vaccines compared with control. GMTs were statistically significantly lower for an HPV-16/18/31/45 AS04 vaccine containing

Abbreviations: AE, adverse event; ANOVA, analysis of variance; ATP, according-to-protocol; CD40L, CD40 ligand; CI, confidence interval; CIN, cervical intraepithelial neoplasia; Ctrl, control; ELISA, enzyme-linked immunosorbent assay; EU, ELISA units; GMT, geometric mean antibody titer; HPV, human papillomavirus; IFN, interferon; IL, interleukin; LU, Luminex units; M, months; MLI, multiplex Luminex immunoassay; MPL, 3-O-desacyl-4'-monophosphoryl lipid A; No., number; PBNA, pseudovirion-based neutralization assay; PCR, polymerase chain reaction; QS21, *Quillaja saponaria* Molina fraction 21; SAE, serious adverse event; TNF, tumor necrosis factor; VLP, virus-like particle.

* Corresponding author. Tel.: +32 3 820 25 38; fax: +32 3 820 26 40.

E-mail addresses: pierre.vandamme@ua.ac.be (P. Van Damme), geert.lerouxroels@ugent.be (G. Leroux-Roels), philippe.simon@erasme.ulb.ac.be (P. Simon), jmfoidart@ulg.ac.be (J.-M. Foidart), gilbert.donders@femicare.net (G. Donders), karel.hoppenbrouwers@med.kuleuven.be (K. Hoppenbrouwers), Myron.Levin@ucdenver.edu (M. Levin), fabian.s.tibaldi@gsk.com (F. Tibaldi), sylviane.poncelet@gsk.com (S. Poncelet), philippe.moris@gsk.com (P. Moris), francis.dessy@gsk.com (F. Dessy), sandra.giannini@gsk.com (S.L. Giannini), dominique.descamps@gsk.com (D. Descamps), Gary.O.Dubin@gsk.com (G. Dubin).

¹ Present address: Laboratory of Tumor Biology and Development, University of Liège, Pathology Tower +4 (B23), Sart-Tilman, B-4000 Liège, Belgium.

² Other institution: University Hospital Antwerpen, Wilrijkstraat 10, 2650 Antwerpen, Belgium.

20/20/10/10 μg VLPs for both anti-HPV-16 and anti-HPV-18 antibodies, and for an HPV-16/18/33/58 AS04 vaccine containing 20/20/20/20 μg VLPs for anti-HPV-16 antibodies. There was also a trend for lower HPV-16 and -18-specific memory B-cell responses for tetravalent AS04 vaccines versus control. No such trends were observed for CD4⁺ T-cell responses. Immune interference could not always be overcome by increasing the dose of HPV-16/18 L1 VLPs or by using a different adjuvant system. All formulations had acceptable reactogenicity and safety profiles. Reactogenicity in the 7-day post-vaccination period tended to increase with the introduction of additional VLPs, especially for formulations containing AS01.

Conclusions: HPV-16 and -18 antibody responses were lower when additional HPV L1 VLPs were added to the HPV-16/18 AS04-adjuvanted vaccine. Immune interference is a complex phenomenon that cannot always be overcome by changing the antigen dose or adjuvant system.

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1. Introduction

More than 500,000 new cases of invasive cervical cancer are diagnosed each year worldwide, resulting in approximately 275,000 deaths [1]. Persistent infection with an oncogenic human papillomavirus (HPV) type is a prerequisite for the development of cervical cancer [2]. Approximately 70% of cervical cancer cases worldwide are associated with HPV-16 and/or HPV-18 [3,4]. Other common oncogenic HPV types associated with cervical cancer include HPV-31, -33, -35, -45, -52 and -58 [4,5,6].

Two prophylactic HPV vaccines against cervical cancer are currently licensed: the HPV-16/18 AS04-adjuvanted vaccine (*Cervarix*[®]) and the HPV-6/11/16/18 vaccine (*Gardasil*[®])³, both consisting of virus-like particles (VLPs) composed of L1 major capsid proteins. In clinical trials, these vaccines had high protective efficacy against persistent infection and cervical intraepithelial neoplasia (CIN) associated with HPV-16/18 and some oncogenic non-vaccine HPV types [7–10]. Moreover, regardless of HPV type in the lesion, the HPV-16/18 AS04-adjuvanted vaccine reduced the incidence of CIN3+ by 93% in women who were HPV-naïve at baseline [11].

Prophylactic vaccines which include additional oncogenic HPV L1 VLPs should theoretically broaden the protection against cervical and possibly other cancers. However, the challenge of developing such vaccines is to ensure that immunogenicity and efficacy against HPV-16/18 (the two most prevalent types in cervical cancer) are not compromised by the introduction of additional HPV L1 VLPs, and that the safety profile and number of doses required are still acceptable. Herein we report the results of two studies evaluating the immunogenicity and safety of two investigational tetravalent HPV L1 VLP vaccines (HPV-16/18/31/45 and HPV-16/18/33/58 vaccines). In these two studies, varying dosages of HPV L1 VLPs (10, 20 or 30 μg), adjuvant systems (AS04, AS01 or AS02 [12,13]) and dosing regimens (0,1,6 months or 0,3 months or 0,6 months) were evaluated.

2. Materials and methods

2.1. Study design

We report data from two separate clinical trials of investigational tetravalent HPV vaccines. In both trials, the licensed HPV-16/18 AS04-adjuvanted vaccine (*Cervarix*[®]), containing 20 μg of each L1 VLP, was used as a control. The amounts of HPV L1 VLPs, formulations and dosing intervals used for the investigational tetravalent vaccines are summarized in Table 1.

Study TETRA-051 (NCT00231413) was a Phase I/II, double-blind, randomized, controlled, dose-ranging trial evaluating an AS04-adjuvanted HPV-16/18/31/45 vaccine, conducted at 11 centers in Belgium and the USA between March 2005 and August 2009. Subjects were randomized (2:1:1:1:1:1) to receive control vaccine or one of 6 different formulations of tetravalent vaccine containing different amounts of HPV L1 VLPs at months (M) 0,1,6. Subjects were initially followed for 6 months after the last vaccine dose (Month 12) in a blinded fashion, after which they were invited to participate in an open-label follow-up study to Month 48.

Study NG-001 (NCT00478621) was a phase I/II, partially blind, randomized, controlled trial evaluating an HPV-16/18/33/58 vaccine, conducted at 3 centers in Belgium between May 2007 and October 2008. Subjects were randomized (1:1:1:1:1) to receive control vaccine at M0,1,6 or one of 5 different formulations/dose schedules of tetravalent vaccine: (i) one formulation with the same concentration of HPV L1 VLPs (20 μg each) and adjuvant system (AS04) as the control vaccine; (ii) two formulations with new adjuvant systems (AS01 and AS02) and containing half the amount of HPV-33 and -58 L1 VLPs (10 μg each) while maintaining the same amount of HPV-16 and -18 L1 VLPs (20 μg each); (iii) finally the AS01 formulation was also tested using two different 2-dose schedules: classic 2-dose (M0,6) or accelerated 2-dose (M0,3). Subjects were followed for 6 months after the last vaccine dose. The trial was open with regard to dose schedule (2-dose or 3-dose) and was observer-blind within the 3-dose groups. Syringes were prepared and administered by qualified medical personnel not otherwise involved in the conduct of the study or in the assessment of symptoms.

For both trials the randomization list was generated at GlaxoSmithKline Biologicals SA using a standard Statistical Analysis System program; a randomization blocking scheme was used to ensure that balance was maintained. Vaccine allocation at all sites was performed using a central randomization call-in system on Internet.

Trials were approved by the appropriate Independent Ethics Committee for each center and carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). Written informed consent was obtained from subjects prior to the performance of any study-specific procedures, after the nature and consequences of the trial had been fully explained.

2.2. Participants

Healthy women aged 18–25 years at the time of first vaccination who had had no more than 6 lifetime sexual partners were eligible for each trial. Subjects of childbearing potential had to have used adequate contraception for 30 days prior to vaccination, have a negative pregnancy test, and continue contraceptive precautions for 2 months after completion of the vaccination series. Other standard eligibility criteria are detailed in the ClinicalTrials.gov registry.

³ *Cervarix* is a registered trade mark of the GlaxoSmithKline group of companies. *Gardasil* is a registered trade mark of Merck & Co., Inc.

Table 1
Vaccine antigen content, adjuvant and dosing schedule.

Group	Group description	Adjuvant system	Dose schedule	Dose interval	Amount of each HPV L1 VLP (μg)					
					16	18	31	45	33	58
Study TETRA-051 (NCT00231413)										
Control	Cervarix [®]	AS04	3-dose	M0,1,6	20	20	–	–	–	–
A	HPV-16/18/31/45 (20/20/10/10 μg) AS04 M0,1,6	AS04	3-dose	M0,1,6	20	20	10	10	–	–
B	HPV-16/18/31/45 (20/20/20/20 μg) AS04 M0,1,6	AS04	3-dose	M0,1,6	20	20	20	20	–	–
C	HPV-16/18/31/45 (20/30/10/10 μg) AS04 M0,1,6	AS04	3-dose	M0,1,6	20	30	10	10	–	–
D	HPV-16/18/31/45 (20/30/20/20 μg) AS04 M0,1,6	AS04	3-dose	M0,1,6	20	30	20	20	–	–
E	HPV-16/18/31/45 (30/20/10/10 μg) AS04 M0,1,6	AS04	3-dose	M0,1,6	30	20	10	10	–	–
F	HPV-16/18/31/45 (30/20/20/20 μg) AS04 M0,1,6	AS04	3-dose	M0,1,6	30	20	20	20	–	–
Study NG-001 (NCT00478621)										
Control	Cervarix [®]	AS04	3-dose	M0,1,6	20	20	–	–	–	–
G	HPV-16/18/33/58 (20/20/20/20 μg) AS04 M0,1,6	AS04	3-dose	M0,1,6	20	20	–	–	20	20
H	HPV-16/18/33/58 (20/20/10/10 μg) AS01 M0,1,6	AS01	3-dose	M0,1,6	20	20	–	–	10	10
I	HPV-16/18/33/58 (20/20/10/10 μg) AS02 M0,1,6	AS02	3-dose	M0,1,6	20	20	–	–	10	10
J	HPV-16/18/33/58 (20/20/10/10 μg) AS01 M0,3	AS01	2-dose	M0,3	20	20	–	–	10	10
K	HPV-16/18/33/58 (20/20/10/10 μg) AS01 M0,6	AS01	2-dose	M0,6	20	20	–	–	10	10

M, month.

2.3. Vaccines

All vaccines were developed and manufactured by GlaxoSmithKline Biologicals SA. The AS04 adjuvant system contains 3-*O*-desacyl-4'-monophosphoryl lipid A (MPL; 50 μg) adsorbed on aluminum salt (500 μg Al³⁺). AS04-adjuvanted vaccines were provided as a liquid suspension in individual pre-filled syringes for single use (0.5 mL). AS01_E is an adjuvant system containing 25 μg MPL, 25 μg *Quillaja saponaria* Molina fraction 21 (QS21) and liposome. AS02_W is an adjuvant system containing 25 μg MPL and 25 μg QS21 in an oil-in-water emulsion. For AS01 and AS02 vaccines, the HPV L1 VLPs were provided as a lyophilized pellet which was reconstituted with 0.5 mL adjuvant immediately prior to administration.

All vaccines were administered (0.5 mL) into the deltoid muscle of the non-dominant arm according to the dosing schedule shown in Table 1.

2.4. Immunogenicity evaluation

The primary objective of each trial was to evaluate antibody responses to HPV-16 and -18 one month after the last vaccine dose. A secondary objective was to evaluate antibody responses to other vaccine HPV types (HPV-31/45 or HPV-33/58). Exploratory objectives were to evaluate cross-reactive antibodies to other non-vaccine HPV types and cell-mediated immunity to vaccine HPV types.

Blood samples for assessment of antibody responses were drawn at Month 0, one month after each vaccine dose, and 6 months after the last vaccine dose. In Study TETRA-051 blood samples were also drawn during the open-label follow-up at Months 18, 24, 36 and 48. In both studies, additional blood samples were drawn from a subset of subjects at pre-selected study sites for assessment of cell-mediated immunity. Assays were done at GlaxoSmithKline Biologicals' laboratories, Rixensart, Belgium.

Quantitation of anti-HPV-16, -18, -31 and -45 antibodies by enzyme-linked immunosorbent assay (ELISA) and pseudovirion-based neutralization assay (PBNA) was based on previously described methodology [14,15]. Multiplex Luminex immunoassay (MLIA) for the simultaneous measurement of anti-HPV-16, -18, -31, -33, -45, -52 and -58 antibodies is described in Supplementary Methods.

Memory B-cell frequencies were measured by B-cell ELISPOT [16]. HPV-specific CD4⁺ T-cells were identified as those expressing two or more immune markers among CD40 ligand (CD40L),

interleukin 2 (IL2), tumor necrosis factor alpha (TNF α) and interferon gamma (IFN γ) after short term in vitro stimulation with HPV type-specific L1 VLPs; frequencies were measured by flow cytometry [17].

2.5. Baseline HPV DNA status

Cervical samples were collected prior to first vaccination to assess baseline HPV DNA status by polymerase chain reaction (PCR), using SPF₁₀ primers and a reverse hybridization line probe assay (LiPA₂₅ version1 manufactured by Labo Biomedical Product, Rijswijk, the Netherlands based on licensed Innogenetics technology) [18].

2.6. Reactogenicity and safety

Solicited local symptoms (pain, redness, or swelling at injection site) and general symptoms (fever, headache, fatigue, gastrointestinal symptoms, arthralgia, myalgia, rash or urticaria) occurring within 7 days after each vaccination were recorded by the subject using a diary card. Investigators documented the presence/absence of urticaria/rash within 30 min after each vaccine dose. Unsolicited adverse events (AEs) occurring within one month of each vaccination, serious adverse events (SAEs), other medically significant conditions (AEs prompting emergency room or physician visits that were not related to common diseases), new onset chronic diseases including new onset autoimmune diseases [16], and pregnancies were documented by the investigator.

2.7. Statistical methods

In each study, the total vaccinated cohort included all vaccinated subjects for whom data were available. The according-to-protocol (ATP) immunogenicity cohort included all evaluable subjects (i.e., those meeting all eligibility criteria, complying with the procedures defined in the protocol, with no elimination criteria during the study) for whom immunogenicity data were available. The primary ATP immunogenicity cohort was defined at the end of the active phase of each study (one month after the last vaccine dose). Secondary ATP immunogenicity cohorts were defined for subsequent time points.

Seropositivity rates with 95% confidence intervals (CIs) and geometric mean antibody titers (GMTs) with 95% CIs were calculated. Summaries were stratified by baseline serostatus. GMTs were calculated by taking the anti-log of the mean of the log titer

Table 2
Demographic characteristics (total vaccinated cohort).

Group	Study TETRA-051: evaluation of HPV-16/18/31/45 vaccine (NCT00231413)										Study NG-001: evaluation of HPV-16/18/33/58 vaccine (NCT00478621)									
	Control	A	B	C	D	E	F	Total	Control	G	H	I	J	K	Total					
Number vaccinated	88	50	49	48	48	50	50	383	90	92	89	90	89	90	540					
Age, years	20.9 (2.03)	21.5 (2.18)	21.2 (2.07)	21.1 (2.09)	21.4 (2.27)	21.2 (2.26)	20.7 (1.91)	21.1 (2.10)	21.0 (2.10)	20.9 (2.02)	21.0 (1.90)	21.1 (1.84)	21.1 (1.84)	21.4 (2.09)	21.1 (2.02)					
Mean (SD)																				
Race, n (%)	1 (1)	0 (0)	1 (2)	1 (2)	1 (2)	1 (2)	1 (2)	6 (2)	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)	0 (0)	1 (<1)					
Black	80 (91)	50 (100)	46 (94)	46 (96)	42 (88)	44 (88)	47 (94)	355 (93)	87 (97)	89 (97)	83 (93)	89 (99)	85 (96)	87 (97)	520 (96)					
White Caucasian/European	4 (5)	0 (0)	1 (2)	1 (2)	3 (6)	1 (2)	0 (0)	10 (3)	1 (1)	2 (2)	1 (1)	1 (1)	2 (2)	1 (1)	8 (1)					
Asian	3 (3)	0 (0)	1 (2)	0 (0)	2 (4)	4 (4)	2 (4)	12 (3)	2 (2)	1 (1)	4 (4)	0 (0)	2 (2)	2 (2)	11 (2)					
Other ^a																				

Control: HPV-16/18 AS04-adjuncted vaccine M0.1.6 (Cervarix®); Group A: HPV-16/18/31/45 (20/20/10/10 µg) AS04 M0.1.6; Group B: HPV-16/18/31/45 (20/20/20/20 µg) AS04 M0.1.6; Group C: HPV-16/18/31/45 (20/30/10/10 µg) AS04 M0.1.6; Group D: HPV-16/18/31/45 (20/30/20/20 µg) AS04 M0.1.6; Group E: HPV-16/18/31/45 (30/20/10/10 µg) AS04 M0.1.6; Group F: HPV-16/18/33/58 (20/20/10/10 µg) AS02 M0.1.6; Group G: HPV-16/18/33/58 (20/20/10/10 µg) AS01 M0.1.6; Group H: HPV-16/18/33/58 (20/20/10/10 µg) AS01 M0.1.6; Group I: HPV-16/18/33/58 (20/20/10/10 µg) AS01 M0.1.6; Group J: HPV-16/18/33/58 (20/20/10/10 µg) AS01 M0.1.6; Group K: HPV-16/18/33/58 (20/20/10/10 µg) AS01 M0.1.6.

^a The "other" race category included Arabic/North African, American Hispanic and mixed race.

transformations. Antibody titers below the cut-off of the assay were given an arbitrary value of half the cut-off for the purpose of GMT calculation.

In TETRA-051, the planned sample size was 376 subjects to give 280 subjects evaluable for immunogenicity (35 subjects for each tetravalent vaccine and 70 subjects for control). This gave at least 80% power to detect a 2.5-fold difference in HPV-16 or HPV-18 GMTs by ELISA one month after the last vaccine dose (primary endpoint). Inferential comparisons of GMTs were made using all subjects in the ATP immunogenicity cohort. The 6 tetravalent vaccine groups were compared using a two-way analysis of variance (ANOVA) F-test model including Factor A (20/20 µg, 30/20 µg or 20/30 µg dose of HPV-16/18), Factor B (10/10 µg or 20/20 µg dose of HPV-31/45) and the interaction between A and B. If a statistical difference was found ($p < 0.025$), pair-wise comparisons were to be made between the 6 groups using Tukey's multiple comparison adjustment. The GMTs of the groups in the factorial design which were not significantly different from the group with the highest HPV-16/18 GMTs were ranked according to dose and compared in sequential order (groups A, E, C, B, F, D) with the control until GMTs in the control group were not significantly higher than the test group. HPV-31/45 GMTs were analyzed in a similar way.

In NG-001, the planned sample size was 540 subjects to give 456 subjects evaluable for immunogenicity (76 subjects per group). This gave 94% power to detect a 2.5-fold difference in HPV-16 or HPV-18 GMTs by ELISA (primary endpoint) between any of the 6 vaccine groups one month after the last vaccine dose. Inferential comparisons of GMTs were done on a subcohort of subjects in the ATP immunogenicity cohort who were initially seronegative and HPV DNA negative at baseline for the corresponding HPV type. The 6 different vaccine groups were compared using a one-way ANOVA F-test. If a statistical difference was found ($p < 0.025$), pair-wise comparisons were made using Tukey's multiple comparison adjustment. Similar analyses were done for GMTs measured by MLIA.

The percentage of subjects with solicited or unsolicited symptoms after each vaccine dose and overall was calculated with exact 95% CI.

3. Results

3.1. Trial population

In TETRA-051, 383 women were vaccinated, 348 (91%) completed the active phase of the study to one month after the last vaccine dose, and 297 (78%) were included in the primary ATP immunogenicity cohort. In NG-001, 540 women were vaccinated, 536 (99%) completed the active phase of the study to one month after the last vaccine dose, and 514 (95%) were included in the primary ATP immunogenicity cohort. Reasons for withdrawal from each study and for exclusion from the ATP immunogenicity cohorts are shown in Fig. 1. In both studies, the mean age of participants was 21 years and the majority ($\geq 93\%$) were of White Caucasian/European ethnic heritage (Table 2).

3.2. Antibody responses for HPV-16 and -18

In both studies, all women were seropositive for anti-HPV-16 and -18 antibodies one month after the last vaccine dose, as measured by ELISA, and remained seropositive through the last assessment (Month 48 for TETRA-051 and Month 12 for NG-001). However, there was a consistent trend for lower anti-HPV-16 and -18 GMTs one month after the last vaccine dose when HPV-31/45 or HPV-33/58 L1 VLPs were added to the HPV-16/18 AS04 vaccine (Fig. 2A and B, respectively). For all vaccines, antibody titers were

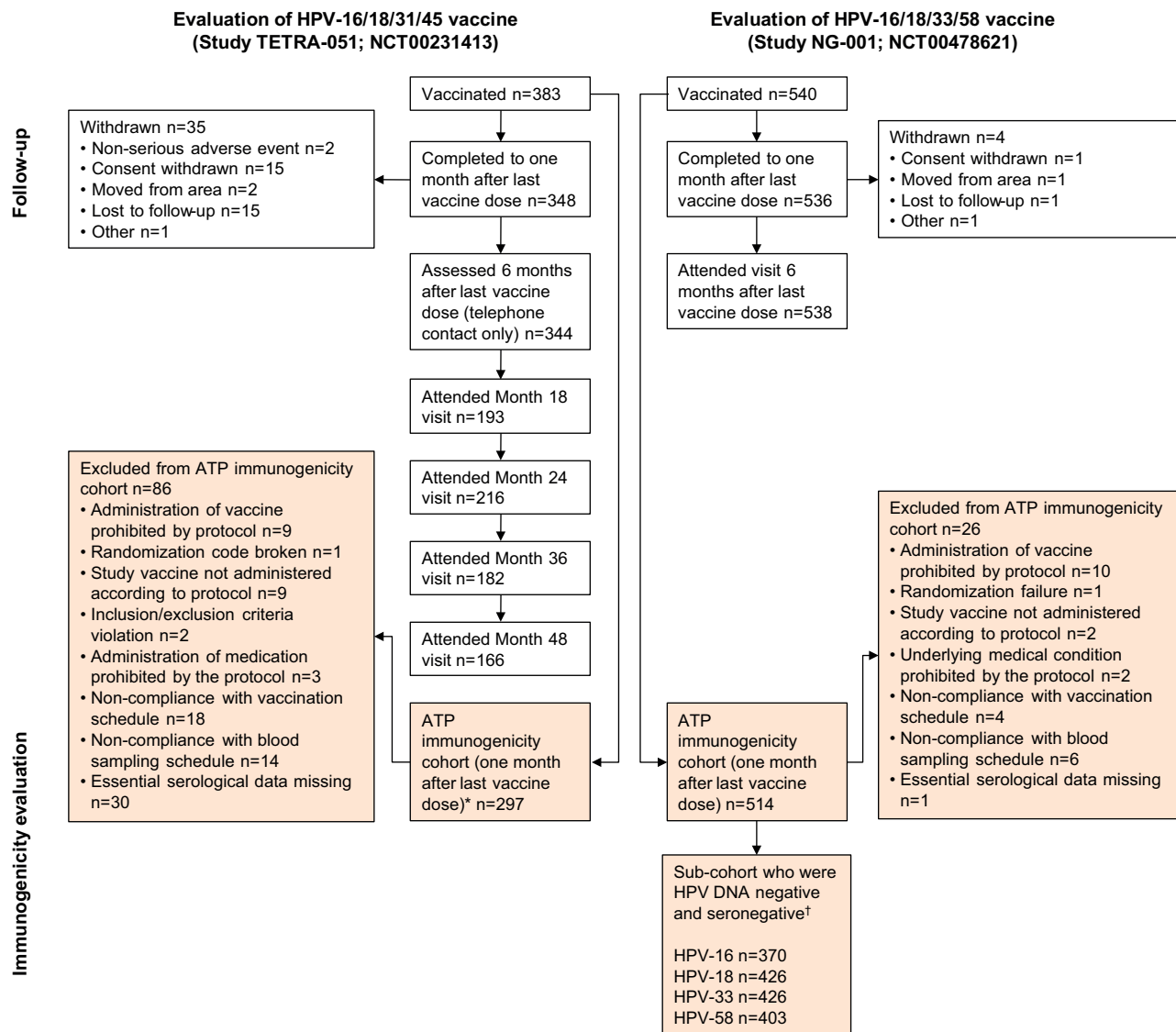


Fig. 1. Flow of participants through each study. ATP, according-to-protocol. * For Study TETRA-051, the main cohort for immunogenicity analyses included all subjects in the ATP immunogenicity cohort irrespective of initial serostatus or HPV DNA status. [†] For Study NG-001, the main cohort for immunogenicity analyses included subjects in the ATP immunogenicity cohort who were initially seronegative for HPV antibodies and HPV DNA negative (by PCR) for the corresponding HPV type and the corresponding assay.

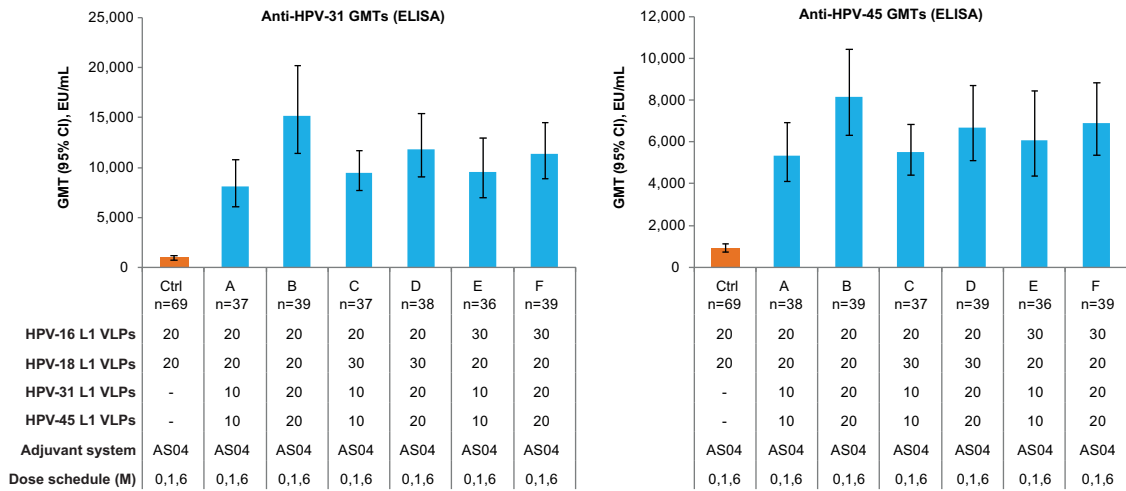
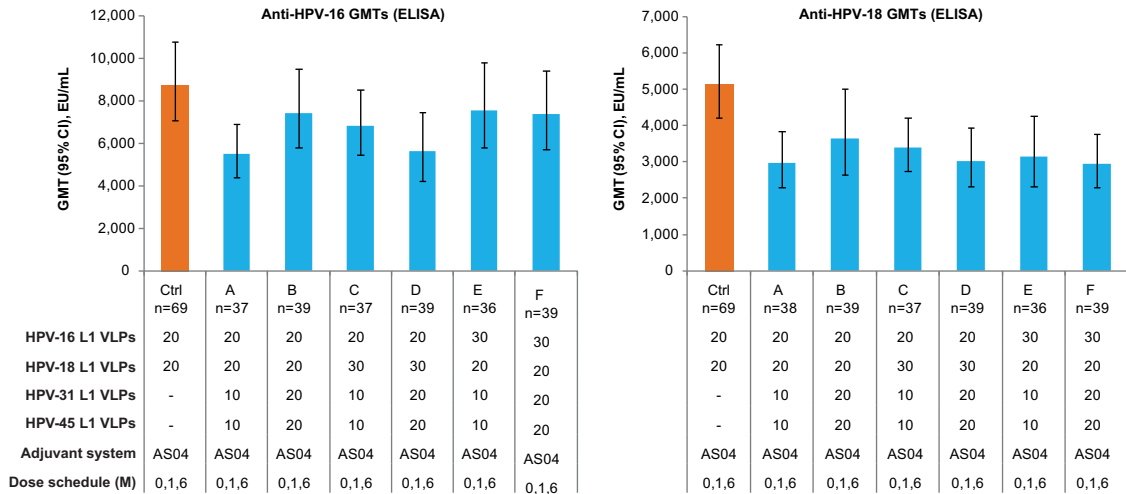
well above those associated with natural infection (i.e., 29.8 ELISA units [EU]/mL for anti-HPV-16 and 22.6 EU/mL for anti-HPV-18) [19].

In TETRA-051, there was no statistically significant difference between the 6 treatment groups in the semi-factorial design in terms of anti-HPV-16 GMTs ($p=0.3377$) or -18 GMTs ($p=0.8364$). In pairwise comparisons, GMTs were significantly lower for group A receiving HPV-16/18/31/45 AS04 (20/20/10/10 μ g) compared with control for anti-HPV-16 antibodies (5505 [95% CI: 4386, 6910] versus 8742 [7075, 10,801] EU/mL; $p=0.0148$) and anti-HPV-18 antibodies (2963 [2287, 3840] versus 5134 [4229, 6234] EU/mL; $p=0.0010$) (Supplementary Table 1). For anti-HPV-16 GMTs, when the amount of HPV-16 L1 VLP was increased from 20 μ g to 30 μ g (group E: 30/20/10/10 μ g), there was no statistically significant difference versus control (7555 [5818, 9811] EU/mL; $p=0.4032$), therefore, no further comparisons were made. For anti-HPV-18 GMTs, when the amount of HPV-18 L1 VLP was increased from 20 μ g to 30 μ g (group C: 20/30/10/10 μ g), the difference versus control was still statistically significant (3406 [2757, 4208] EU/mL; $p=0.0086$). When the amount of HPV-31/45 VLPs was increased

from 10 μ g to 20 μ g (group B: 20/20/20/20 μ g), anti-HPV-18 GMTs were still lower versus control but not statistically different (3643 [2640, 5027] EU/mL; $p=0.0540$).

In Study NG-001, in women who were initially seronegative and HPV DNA negative for the corresponding HPV type, significantly lower anti-HPV-16 GMTs were observed for the HPV-16/18/33/58 AS04 vaccine containing 20 μ g of each L1 VLP compared with control (6775 [5502, 8342] versus 11,246 [9133, 13,847] EU/mL; $p=0.0017$) (Supplementary Table 1). However, anti-HPV-16 GMTs were significantly higher for the 3-dose tetravalent vaccine adjuvanted with AS01 (27,645 [22,713, 33,649] EU/mL; $p<0.0001$) or AS02 (17,664 [14,534, 21,468] EU/mL; $p=0.0055$) compared with control. Lower anti-HPV-18 GMTs were observed for the HPV-16/18/33/58 AS04 vaccine containing 20 μ g of each L1 VLP compared with control, although this difference was not statistically significant (2987 [2428, 3675] versus 4332 [3521, 5330] EU/mL; $p=0.0513$) (Supplementary Table 1). Anti-HPV-18 GMTs were still lower than control even when different adjuvant systems were used, though the 3-dose AS01 vaccine elicited the best anti-HPV-18 response out of the various tetravalent vaccine

A. Study TETRA-051



B. Study NG-001

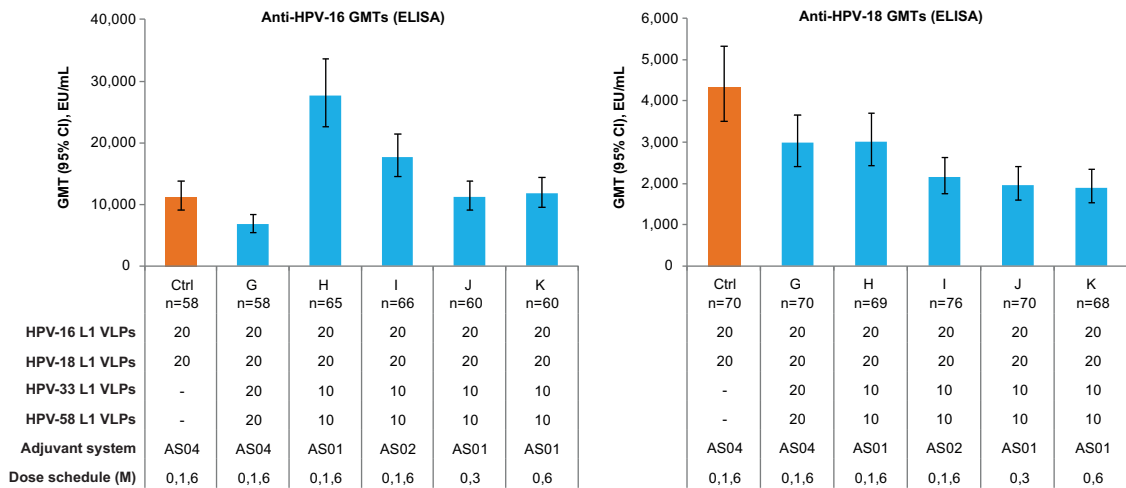


Fig. 2. Antibody titers against vaccine types one month after last vaccine dose by ELISA (ATP immunogenicity cohort). Bars show geometric mean antibody titer (GMT) and corresponding 95% confidence interval (CI) for each vaccine, determined by enzyme-linked immunosorbent assay (ELISA), one month after the last vaccine dose (Month 7 for M0,1,6 or M0,6 schedules and Month 4 for M0,3 schedule). Amounts of HPV L1 virus-like particles (VLPs) are in μg . Ctrl, control (HPV-16/18 AS04-adjuvanted vaccine). Data are shown for the main cohort for inferential analysis for each study, i.e., all subjects in the ATP immunogenicity cohort irrespective of initial serostatus or HPV DNA status for Study TETRA-051 (panel A), and subjects in the ATP immunogenicity cohort who were initially seronegative for HPV antibodies and HPV DNA negative (by PCR) for the corresponding HPV type for Study NG-001 (panel B). Note that anti-HPV-33 and anti-HPV-58 titers were not determined by ELISA in Study NG-001.

formulations tested. Anti-HPV-16 and -18 GMTs were significantly lower one month after the last vaccine dose when 2 doses (M0,3 or M0,6) of the AS01 formulation were administered, compared with 3 doses of the same AS01 formulation.

The results obtained for neutralizing antibodies measured by PBNA in a subset of subjects (Supplementary Fig. 1) were generally in line with those from ELISA testing, although numbers of subjects evaluated were small.

3.3. Antibody responses for non-HPV-16/18 vaccine types

In TETRA-051 (Fig. 2A), there was a significant impact of the HPV-31/45 dose on anti-HPV-31 and -45 GMTs. For groups with a 20 µg dose of HPV-31 and -45 L1 VLPs (groups B, D and F combined), the estimated anti-HPV-31 GMT one month after the last vaccine dose was approximately 1.4-fold higher than for groups with a 10 µg dose (groups A, C and E combined) (12,667 [10,907, 14,711] versus 9173 [7867, 10,696] EU/mL; $p=0.0033$) and the estimated anti-HPV-45 GMT was approximately 1.3-fold higher (7214 [6237, 8345] versus 5638 [4855, 6548] EU/mL; $p=0.0209$). All tetravalent vaccine formulations elicited anti-HPV-31 and anti-HPV-45 GMTs that were at least 44-fold higher and 38-fold higher, respectively, than those associated with natural infection (i.e., 183.5 EU/mL for anti-HPV-31 and 139.0 EU/mL for anti-HPV-45) [20].

In NG-001 (Supplementary Table 1), in women who were initially seronegative and HPV DNA negative for the corresponding HPV type, anti-HPV-33 GMTs were significantly higher one month after the last vaccine dose for the 3-dose AS01 vaccine (21,505 [17,842, 25,920] LU/mL) compared with AS02 (12,963 [10,846, 15,493] LU/mL, $p=0.0001$) or AS04 (7102 [5869, 8595] LU/mL, $p<0.0001$), with half the HPV-33/58 VLP content of the AS04 tetravalent formulation. Anti-HPV-58 GMTs were also significantly higher for the 3-dose tetravalent vaccine adjuvanted with AS01 (10,897 [9090, 13,064] LU/mL) compared with AS02 (6925 [5805, 8261] LU/mL, $p=0.0006$) or AS04 (5524 [4556, 6698] LU/mL, $p<0.0001$), with half the HPV-33/58 VLP content of the AS04 tetravalent formulation. For the AS01 formulation, anti-HPV-33 and -58 GMTs were significantly lower one month after the last vaccine dose when 2 doses (M0,3 or M0,6) were administered, compared with 3 doses.

3.4. Cross-reactive antibodies

In Study NG-001, all tetravalent vaccine formulations produced cross-reacting anti-HPV-31, anti-HPV-45 and anti-HPV-52 GMTs which were at least 4-fold, 7-fold and 3-fold higher, respectively, than those associated with natural infection (i.e., 61.6 LU/mL for anti-HPV-31, 28.7 LU/mL for anti-HPV-45 and 54.5 LU/mL for anti-HPV-52). The 3-dose tetravalent HPV-16/18/33/58 vaccine adjuvanted with AS01 induced higher levels of cross-reacting antibodies to non-vaccine antigens (HPV-31, -45 and -52) one month after the last vaccine dose than vaccines adjuvanted with AS02 or AS04 (Supplementary Fig. 2). Cross-reacting antibody responses tended to be lower when the HPV-16/18/33/58 AS01 vaccine was administered on a 2-dose schedule than a 3-dose schedule.

3.5. Memory B-cell responses

In TETRA-051 (Fig. 3A), all vaccines induced similar frequencies of HPV-16 and -18 specific memory B-cells one month after the last vaccine dose, but the frequencies of HPV-31 and -45 specific memory B-cells were higher in tetravalent HPV-16/18/31/45 vaccine groups than in the control group, regardless of VLP concentration (median HPV-31 specific B-cell counts per 10^6 B-cells [interquartile range] ranged from 2203 [1042–7567] to 5374 [2510–7642] for tetravalent formulations versus 263 [194–922] for

control, and median HPV-45 specific B-cell counts ranged from 683 [437–2935] to 2246 [760–7538] for tetravalent formulations versus 198 [100–567] for control).

In Study NG-001 (Fig. 3B), the median frequency of HPV-16 specific memory B-cells one month after the last vaccine dose was approximately 2-fold lower for the tetravalent AS04 vaccine (729 [563–1484]) than control (1518 [865–2588]), whereas tetravalent vaccines adjuvanted with AS01 (4550 [2117–7031]) and AS02 (2950 [1384–5014]) induced higher median frequencies of HPV-16 specific B-cells than control. The median frequency of HPV-18 specific B-cells was approximately 1.6-fold lower for the tetravalent AS04 vaccine (512 [113–1312]) and 1.5-fold lower for the AS02 vaccine (533 [211–1139]) than control (818 [416–2134]), whereas the AS01 vaccine (919 [430–1493]) induced similar median frequencies of HPV-18 specific memory B-cells to control. The tetravalent formulations induced higher frequencies of HPV-33 and -58 specific B-cells, compared to cross-reacting HPV-33 and -58 specific B-cell responses induced by the control vaccine (HPV-33 specific B-cell counts ranged from 1453 [631–3044] to 5678 [2610–8551] for tetravalent formulations versus 124 [39–317] for control, and HPV-58 specific B-cell counts ranged from 1907 [910–2452] to 4006 [2117–5805] for tetravalent formulations versus 112 [34–385] for control). Comparing the tetravalent formulations, the highest median B-cell response for all four vaccine types was induced by the AS01 formulation, regardless of dose schedule; the AS02 formulation induced an intermediate response and the AS04 formulation induced a lower response.

3.6. CD4⁺ T-cell responses

In TETRA-051 (Fig. 4A), the control vaccine induced strong CD4⁺ T-cell responses to both HPV-16 and -18 one month following last vaccination, and induced cross-reacting CD4⁺ T-cell responses to HPV-31 and -45. All tetravalent formulations also induced high levels of CD4⁺ T-cells to HPV-16, -18, -31 and -45, regardless of VLP content.

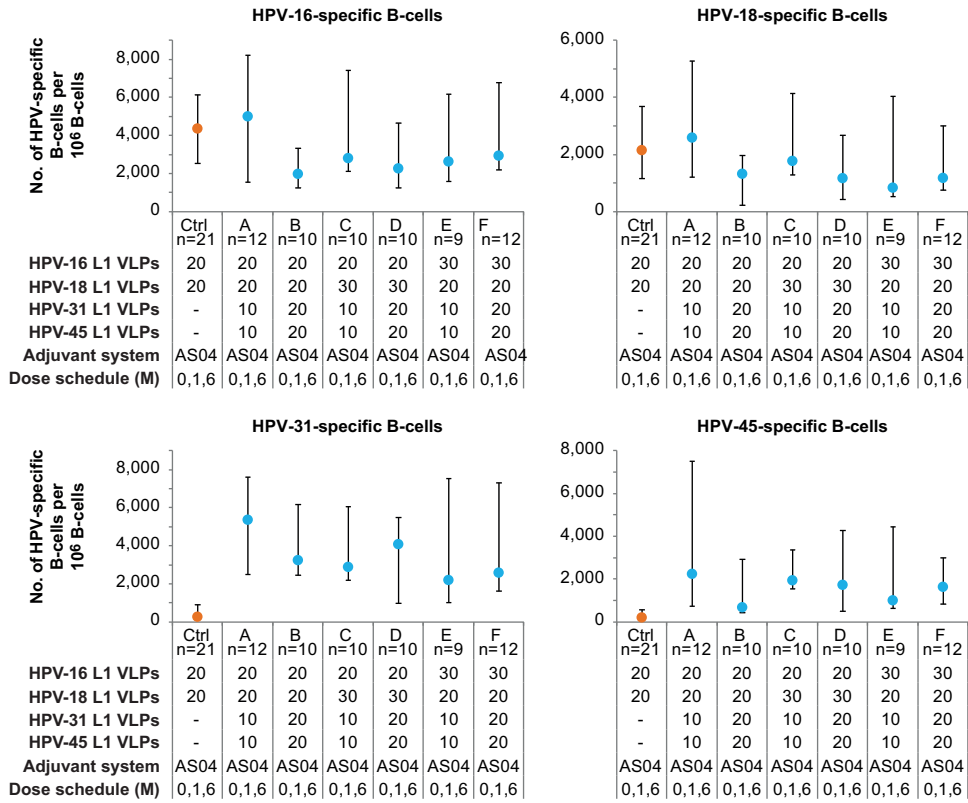
In Study NG-001 (Fig. 4B), one month after the last vaccine dose, median frequencies of HPV-16 specific CD4⁺ T-cells were similar for control and tetravalent vaccines. Median frequencies of HPV-18 specific CD4⁺ T-cells were more than 2-fold lower for each of the tetravalent formulations compared with the control vaccine, although interquartile ranges overlapped. Frequencies of HPV-33 and -58 specific CD4⁺ T-cells induced by the tetravalent vaccine formulations were similar to the frequencies of cross-reactive CD4⁺ T-cells induced by the control vaccine, regardless of adjuvant system, number of doses or VLP content.

3.7. Reactogenicity and safety

In TETRA-051, reactogenicity profiles of the different formulations of the HPV-16/18/31/45 AS04 vaccine were similar across all six groups and were generally comparable to the profile for the control vaccine (Supplementary Figs. 3 and 4). There was, however, a consistent trend for more grade 3 pain in the tetravalent groups (reported following 8.4–14.9% of doses) compared to the control group (reported following 6.1% of doses). Through Month 48, 23 subjects reported non-fatal SAEs (Supplementary Table 2). One SAE, myelitis for a subject in the HPV-16/18/31/45 (20/30/10/10 µg) group, was considered to be possibly related to vaccination by the investigator. There were two withdrawals due to non-serious AEs (pruritus and injection site pain).

In NG-001, there was a trend for increased reactogenicity during the 7-day post-vaccination period for tetravalent formulations compared with control vaccine, particularly for formulations containing AS01 (Supplementary Figs. 3 and 5). Local solicited symptoms were reported following 91.9% of doses for the control

A. Study TETRA-051



B. Study NG-001

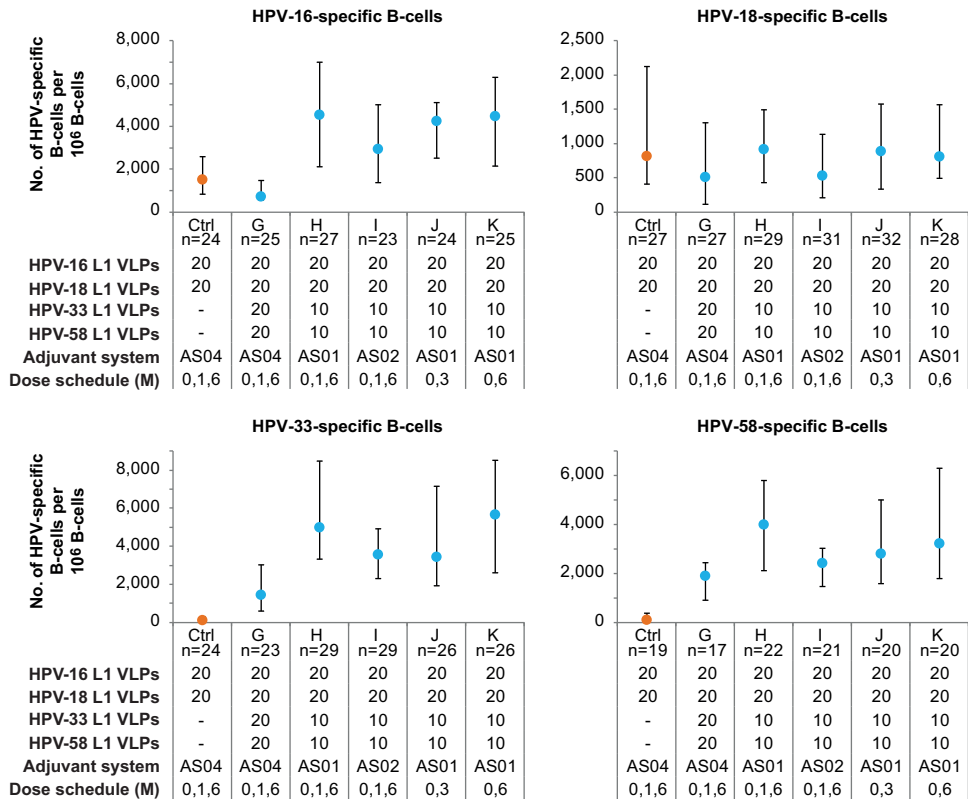
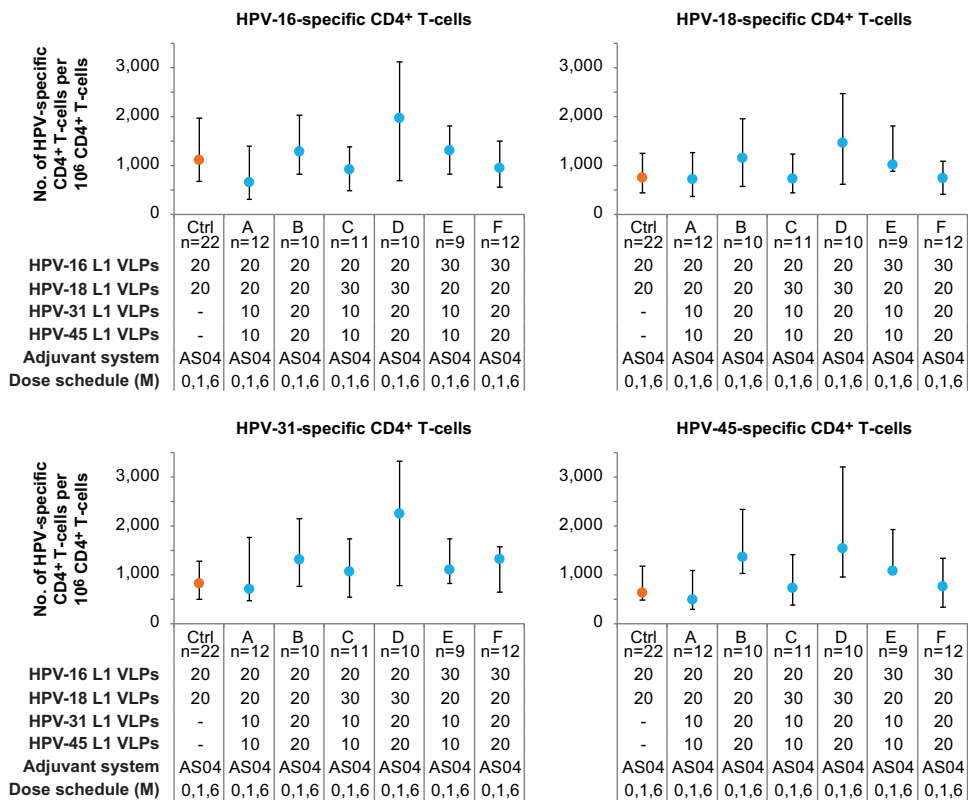


Fig. 3. Median number (interquartile range) of HPV-specific B-cells per 10^6 B-cells one month after last vaccine dose (ATP immunogenicity cohort). Closed circles show the median number of HPV-specific B-cells per 10^6 B-cells. Error bars represent the interquartile range. Data are shown for the main analysis cohort for each study, i.e., all subjects in the ATP immunogenicity cohort irrespective of initial serostatus or HPV DNA status for Study TETRA-051 (panel A), and subjects in the ATP immunogenicity cohort who were initially seronegative for HPV antibodies, HPV DNA negative (by PCR), and B-cell negative for the corresponding HPV type for Study NG-001 (panel B). Ctrl, control (HPV-16/18 AS04-adjuvanted vaccine); No., number.

A. Study TETRA-051



B. Study NG-001

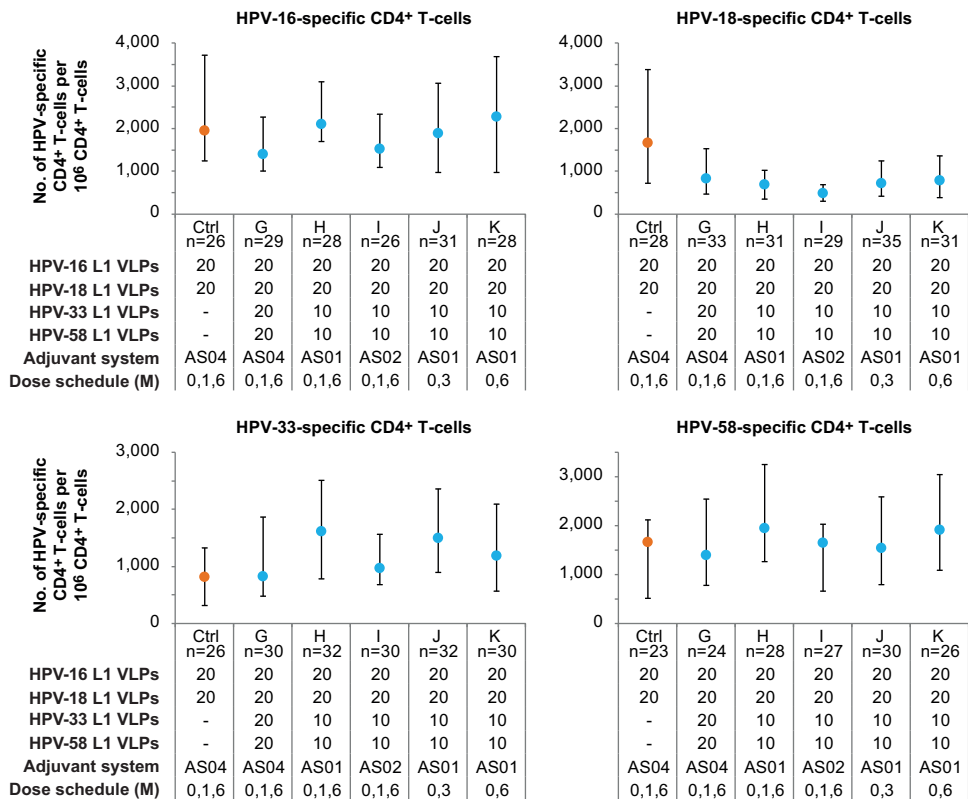


Fig. 4. Median number (interquartile range) of HPV-specific CD4+ T-cells per 10⁶ CD4+ T-cells one month after last vaccine dose (ATP immunogenicity cohort). Closed circles show median number of HPV-specific CD4+ T-cells secreting at least 2 different cytokines per 10⁶ CD4+ T-cells. Error bars represent the interquartile range. Data are shown for the main analysis cohort for each study, i.e., all subjects in the ATP immunogenicity cohort irrespective of initial serostatus or HPV DNA status for Study TETRA-051 (panel A), and subjects in the ATP immunogenicity cohort who were initially seronegative for HPV antibodies and HPV DNA negative (by PCR) at baseline for the corresponding HPV type for Study NG-001 (panel B). Ctrl, control (HPV-16/18 AS04-adjuvanted vaccine); No., number.

group and 95.8–98.3% of doses for AS01 groups. General solicited symptoms were reported following 55.6% of doses for the control group and 68.3–76.1% of doses for AS01 groups. All solicited general symptoms, except rash and urticaria, occurred with higher frequency for the AS01 vaccine than for AS04 or AS02 vaccines (Supplementary Fig. 5). Through Month 12, 12 subjects reported non-fatal SAEs (Supplementary Table 2). None of the SAEs was considered to be possibly related to vaccination by the investigator. There were no withdrawals due to an AE.

There was no recognizable pattern in terms of timing or types of SAEs, other medically significant conditions, or new onset chronic diseases (including new onset autoimmune diseases) reported across the vaccine groups in either study.

4. Discussion

It is well documented that inclusion of additional antigens in non-HPV vaccines can have a positive or negative effect on immunogenicity and reactogenicity [21–26]. In two trials evaluating investigational adjuvanted tetravalent HPV vaccines, we found that new HPV L1 VLPs (HPV-31/45 or HPV-33/58) introduced into the vaccine were immunogenic, but tended to lower the magnitude of anti-HPV-16 and -18 antibody responses, compared with the licensed HPV-16/18 AS04-adjuvanted vaccine. Whether this would result in a reduction in either the quality or duration of protection against HPV infection or other clinical endpoints is not known, since immunological correlates of protection for HPV have not been defined. Thus, care needs to be used interpreting these results.

For anti-HPV-16 antibodies, the immune interference could be overcome by a change in vaccine formulation (either by increasing the dose of HPV-16 L1 VLPs, or by using a different adjuvant system). In fact, a particularly high anti-HPV-16 antibody response was elicited when the tetravalent HPV-16/18/33/58 vaccine was adjuvanted with AS01 or AS02, compared with the control vaccine. This finding was supported by the detection of higher HPV-16 specific memory B-cell responses for formulations containing AS01 and AS02, although these adjuvant systems did not notably impact on HPV-16 specific CD4⁺ T-cell responses. An evaluation of the interaction of specific CD4⁺ T-cell help for memory B-cell maturation and antibody affinity may shed some light on the results observed.

The nature of the negative immune interference with regard to anti-HPV-18 humoral and cellular immunity was more complex and could not always be overcome by increasing the dose of HPV-18 L1 VLPs, or by using a different adjuvant system. Interestingly, we observed that increasing the amount of HPV-31/45 VLPs from 10 µg to 20 µg did improve the anti-HPV-18 immunogenicity of a tetravalent HPV-16/18/31/45 vaccine, although anti-HPV-18 GMTs were still lower than those elicited by the control vaccine. This was presumably because of enhanced induction of cross-reactive HPV-18 antibodies induced by HPV-45 (both are A7 species of HPV).

As expected, we found that specific antibody responses to the additional HPV L1 VLPs introduced in the tetravalent vaccines (HPV-31 and -45 or HPV-33 and -58) were significantly higher compared with cross-reacting antibodies induced by the control vaccine. However, it is not possible to predict from the two studies reported herein whether enhanced immune responses with polyvalent vaccines against a broader range of oncogenic HPV types will translate into higher clinical efficacy than previously reported [11].

Although the precise contribution of HPV-16, -33 and -58 to cross-reactivity against other species of HPV (HPV-31 and HPV-52) cannot be defined, it is clear that adjuvantation with AS01 has a major impact on the cross-reactive behavior of the tetravalent HPV-16/18/33/58 vaccine. A tentative explanation for this relates to the ability of AS01 to stimulate the innate immune response, to enhance or modulate antigen-specific antibody and T cell-mediated

responses [13]. Major type-specific regions on HPV L1 VLPs that are surface exposed and conformation dependent have been identified for a few HPV types, but very little is known about the regions of HPV L1 VLPs important for cross-reactivity [27].

Although the correlates of immunity for HPV remain unknown, it has been demonstrated in seropositive women who cleared an HPV-16 or -18 infection that higher type-specific HPV antibody titers are associated with a reduced risk of re-infection with the same HPV type [28]. It is unknown if antibodies are a surrogate marker for immunity and if this same association will be seen in vaccinated women whose antibody responses are typically much higher than those seen after natural infection. However, it has previously been shown that the HPV-16/18 AS04-adjuvanted vaccine induces cross-neutralizing antibodies that may mediate cross-protection [29]. Further, it has been suggested that the magnitude of the immune response may represent a determinant of duration of protection, although this remains to be proven [16,17,20].

When the HPV-16/18/33/58 AS01 vaccine was administered as a 2-dose regimen, the HPV type-specific antibody response to all HPV antigens tested was lower than when receiving 3 doses of the same formulation. However, the NG-001 study was not designed to formally evaluate non-inferiority of immune responses for different dose schedules, and was performed in an older age group than previous 2-dose studies. It has previously been shown that anti-HPV-16 and -18 antibody levels elicited by 2-dose schedules of the licensed HPV-16/18 AS04-adjuvanted vaccine may be adequate for girls aged 9–14 years [30], however, further investigation is ongoing. Furthermore, in a large Costa Rican trial in women aged 18–25 years it was shown that 2 doses of the HPV-16/18 vaccine were as protective against persistent infection as 3 doses over a 4-year period post-vaccination [31].

Although all tetravalent formulations had an acceptable reactogenicity and safety profile, there was a tendency toward an increase in reactogenicity when additional HPV L1 VLPs were added to the vaccine, especially with formulations containing AS01.

It was not the aim of this paper to directly compare the two studies reported herein. The rationale was to present the results of two separate studies (with different design, number of participants, investigational products, study cohorts, and data sets analyzed) that led to very similar results and support the same observation, i.e., that adding different HPV antigens to the licensed HPV-16/18 AS04-adjuvanted vaccine can cause negative immune interference with regard to HPV-16/18 humoral and/or cellular immunity, although the clinical relevance of this immune interference is unknown. Even though the sub-cohorts of subjects under analysis were not the same, the authors believe that results of both studies, when taken together, strengthen the conclusion on immune interference.

Immune interference is complex and cannot necessarily be overcome by increasing the dose of the affected HPV L1 VLP, or by changing the adjuvant, but may be overcome by altering the relative ratios of the HPV L1 VLP components of the vaccine. When developing a vaccine, consideration should be given to selecting the optimal combination of antigens and adjuvants, taking into account the intended purpose and target population of the vaccine. Although the AS04 adjuvant system is adequate for the bivalent HPV-16/18 vaccine, next-generation polyvalent vaccines may require the use of other adjuvant systems or technologies.

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Conflict of interest: All authors have completed the Unified Competing Interest form at www.icmje.org/coi_disclosure.pdf and declare: P.V.D. acts as (principal) investigator for vaccine trials conducted on behalf of the University of Antwerp, for which the University obtains research grants from vaccines companies. G.L.R. acts as (principal) investigator for vaccine trials conducted on behalf of the Ghent University, for which the University obtains research grants from vaccines companies. P.S. received consulting fees or honorarium for his institution, fees for participation in review activities such as data monitoring boards, statistical analysis, endpoint committees and the like. Ga. Du., K.H., J.M.F. and P.S. received support for travel to meetings for the study. Ga. Du. received reimbursement for travel expenses for business related activities (other than the study). K.H., M.L. and J.M.F. received grants for their institutions. K.H. and P.S. received financial support for board membership. G.L.R., M.L., J.M.F. and P.S. received financial support for consultancy. G.L.R. and P.S. received payment for lectures including service on speaker bureaus. D.D., F.D., Ga. Du., P.M., S.P., F.T. and S.L.G. are GlaxoSmithKline employees. D.D., Ga. Du., P.M., F.T. and S.L.G. have GlaxoSmithKline stock options. Gi. Do. declared no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vaccine.2014.03.040>.

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