Antibody avidity measurements in recipients of Cervarix® vaccine following a two-dose schedule or a three-dose schedule

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A B S T R A C T

The HPV-16/18 vaccine (Cervarix®) is a prophylactic vaccine for the prevention of cervical cancer and contains recombinant virus-like particles (VLPs) assembled from the L1 major capsid proteins of human papillomavirus (HPV) strains 16 and 18. Although a correlate of protection has yet to be identified, HPV-specific antibodies are thought to prevent virus infection of the genital mucosa. Therefore, antigen-specific antibodies as assessed by ELISA or pseudovirion-based neutralisation assay are frequently measured in clinical trials to substantiate the immune responses induced by the vaccine. Measuring antigen-antibody binding avidities, which reflects the degree of affinity maturation in the B-cells, is another valuable method to assess the quality of the antibody responses. Here we describe the antigen-specific antibody avidities in samples taken from a clinical trial examining the feasibility of adopting a two-dose (Months 0 and 6) schedule for 9–14 year olds instead of the three-dose schedule (Months 0, 1 and 6). Antibody avidity (i.e. avidity index [AI]) was determined in the ELISA by the ratio of antibody concentrations in serum samples treated or not with the chaotropic agent NaSCN. Importantly, in the comparison between the groups of two-dose and three-dose recipients, no differences in AIs were observed at Months 7, 24 and 48. The results suggest that from Month 7 to 48, the quality of the antibody response in terms of avidity was similar in the two-dose recipients to that in the three-dose recipients. Hence these results support the adoption of a two-dose schedule in 9–14 year-old girls.

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1. Introduction

Oncogenic strains of the human papillomavirus (HPV) cause cervical cancer [1]; and two particular strains, HPV16 or HPV18, have been identified in over 70% of cervical cancers [2]. The AS04-adjuvanted HPV-16/18 vaccine (Cervarix®; GlaxoSmithKline [GSK] Biologicals SA) is a prophylactic vaccine for the prevention of cervical cancer and contains recombinant virus-like particles (VLPs) assembled from the L1 major capsid proteins of HPV16 and HPV18. The HPV-16/18 vaccine has demonstrated very high efficacy against persistent infections and high-grade lesions associated with HPV-16/18 as well as cross-protective efficacy against other oncogenic HPV such as HPV31 and 45 [3,4]. Overall, the vaccine efficacy against cervical intraepithelial neoplasias graded 3 or greater in a cohort of HPV DNA-negative women has been estimated at 93.2% (95% CI 78.9–98.7), irrespective of HPV type [3].

Since the preferred age range for HPV-16/18 vaccination (9–14 years) is younger than the age range in which efficacy is typically assessed (beyond 16 years), measurement of the concentration and quality of antibody responses in this population is crucial [5,6]. Antibodies are thought to play a role in preventing HPV infection of genital mucosa, even though a correlate of protection has yet to be identified [7,8]. Typical methods for assessing antigen-specific antibody responses include ELISAs of cervical secretions as well as serum, pseudovirion-based neutralisation assays, and measuring the frequencies of memory B cells [9–11]. The avidity ELISA is another measure of the antibody response. Increased antibody avidity for antigens reflects the process of affinity maturation of B cells in the germinal centres that in the presence of follicular helper T cells (Tfh) progressively produce antibodies with higher affinity via somatic hypermutation events and develop into B memory cells or plasma cells [12–14].

Higher avidities of influenza haemagglutinin (HA1)-specific antibodies have been correlated with higher neutralisation titres

Abbreviations: VLP, virus-like particle; AI, avidity index; GM, geometric mean; GMC, geometric mean concentration.
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after a A(H5N1) influenza vaccination schedule where prime and boost injections were 12–24 weeks apart [15]. Similarly, higher antibody avidities have been associated with higher bactericidal activities in the assessment of Haemophilus influenzae type b vaccines [16,17] and Streptococcus pneumoniae type 6B and 23F vaccines [18]. In a recent study of women vaccinated with the HPV-16/18 vaccine, relatively higher levels of HPV16 L1-specific antibodies and avidities were associated with the prevention of HPV31 infection of the cervix [19]. Detailed kinetic measurements of antigen-specific antibody avidities have also been made with the unadjuvanted HPV16-L1 VLP vaccine [20] and the HPV-16/18 vaccine [10], administered in a three-dose schedule (Months 0, 1 and 6); and the increase in antibody avidities in these studies are consistent with a process of sequential affinity maturation [21,22].

Although the HPV-16/18 vaccine is licenced in accordance with a three-dose schedule (Months 0, 1 and 6), a two-dose schedule is under evaluation in clinical trials (Month 0 and 6 or 12). In one recent clinical trial, the feasibility of adopting a two-dose (Month 0 and 6) schedule for 9–14 year olds has been supported on the basis of vaccine-specific antibody responses, as assessed by ELISA and on the basis of safety during 24 months of follow-up [6]. Furthermore, two doses of the vaccine appeared as protective as three doses over the four years of follow-up, in one clinical trial where some vaccine recipients did not complete the three-dose schedule [23].

The aim of this study was to compare the quality of antibody responses in clinical trial recipients of two-doses (Months 0 and 6 in 9–14 year olds) or three-doses (Months 0, 1 and 6 in 15–25 year olds) of the HPV-16/18 vaccine by measuring antigen-specific antibody avidities. An initial step in this study was to characterise a modified ELISA for measuring avidity using the chaotropic agent NaSCN together with samples taken from other clinical trials of the HPV-16/18 vaccine using a three-dose (Months 0, 1 and 6) schedule.

2. Materials and methods

2.1. Study designs and sample collection

In Studies 1 and 2, serum samples were collected at 1-month post-Dose 2 (Month 2) and post-Dose 3 (Month 7) from healthy female human subjects who had received three intramuscular injections (Months 0, 1 and 6) of the HPV-16/18 vaccine from clinical trials NCT00196937 (N = 30, 10–14 years old) and NCT00196937 (N = 35, 15–28 years old; N = 21, 29–41 years old; and N = 34, 42–55 years old) [24,25]. In Study 3, serum samples were collected at 1, 18, or 42-months post-last dose (Months 7, 24 and 48) from healthy human female subjects from clinical trial NCT00541970 who either had received the HPV-16/18 vaccine as two intramuscular injections (Months 0 and 6, N = 30, 9–14 year olds), or three intramuscular injections (Months 0, 1 and 6, N = 30, 15–25 year olds) [6]. The serum samples for the study were randomly selected from what was available in the clinical trial archives and with respect to the trial participants’ identification numbers. All serum samples were stored at −20°C. All trials were approved by research ethics committees of the respective participating countries and conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. Written informed consent was obtained from each trial participant who was at least the age of consent. Written informed assent was obtained from each trial participant below the age of consent in addition to written informed consent from her parent/guardian. One Cervarix® dose contains 20 μg of HPV16 L1 VLP, 20 μg of HPV18 L1 VLP, 50 μg 3-O-desacyl-4-monophosphoryl lipid A (MPL) and 500 μg aluminium hydroxide. All doses were constituted in 0.5 ml.

2.2. Determination of chaotropic agent concentration and avidity ELISA

Ninety-six well plates were coated with HPV16, HPV18, HPV31 or HPV45 L1 VLPs (0.5 to 1.5 μg/ml) overnight at 4°C, and blocked with 1% bovine serum albumin, 0.1% Tween-20 in phosphate-buffered saline.

For the determination of the chaotropic agent concentration, coated wells were incubated with 0–8 M NaSCN for 15 min at room temperature. After a washing step, wells were incubated with biotinylated V5 (1.56 ng/ml; anti-HPV16) or J4 (6.25 ng/ml; anti-HPV18) monoclonal antibodies for 90 min at 37°C.

For the avidity ELISA, coated wells were incubated with serum samples (12 serial 2-fold dilutions) for 1 h 30 min at 37°C. After a washing step, wells were incubated with 0 or 1 M NaSCN for 15 min at room temperature. After another washing step, wells were then incubated with biotin-conjugated anti-human IgG (Jackson; Studies 1 and 2) or Ig (Amersham; Study 3).

Biotinylated antibody detection used the colorimetric readout based on streptavidin–horseradish peroxidase (Amexd, GE Healthcare) and O-phenylenediamine substrate (Sigma). Optical densities were read at 492/620 nm and antibody concentrations were calculated relative to a standard antiserum reference using SoftMaxPro software (4-parameter equation) and expressed in EU/ml. An avidity index (A Phó) was calculated as a ratio of the antigen-specific antibody concentration determined after 1 M NaSCN treatment divided by the antigen-specific antibody concentration without NaSCN treatment.

2.3. Statistics

All statistical analyses were not part of the objectives of the clinical trials from which the samples were taken and therefore were considered as exploratory. Parametric analyses were performed using SAS software on log10 transformed data. The Shapiro–Wilk test, Skewness and Kurtosis calculations were used to confirm normality. Differences were identified by ANOVA followed by Tukey’s test. All comparisons were two-tailed. Pearson’s r statistic was used to identify correlations between (log10 transformed) AIs and antibody concentrations. Significance was ascribed to p-values <0.05 (and in the case of antibody concentrations, to ≥2-fold differences). AIs are described to two-significant figures in the text.

3. Results

3.1. Concentration determination of chaotropic agent

The HPV16 L1 and HPV18 L1 conformational epitopes that are important epitopes for neutralising antibodies [7,26], were evaluated in an ELISA using monoclonal antibodies V5 and J4, respectively. Both epitopes were not significantly denatured by 15 min pre-incubation with <4 M NaSCN (Fig. 1). However, 10% of HPV16 L1 conformational epitopes were denatured by 2 M NaSCN. Therefore, a 15 min incubation with 1 M NaSCN in the ELISA was selected to assess the antibody avidities with serum samples from HPV-16/18 vaccine recipients.

3.2. Comparisons between AIs at Month 2 and Month 7 in a three-dose vaccination schedule (Months 0, 1 and 6)

In Study 1 and 2, the AIs of HPV16 L1- and HPV18 L1-specific antibodies were assessed in samples taken from vaccinated girls and women one month post-Dose 2 (Month 2) and one month post-Dose 3 (Month 7). In Study 1, samples were taken from 10 to 14 year-old girls, whereas in Study 2, samples were taken from 15 to 55 year-old girls and women allocated to three groups stratified
avidity means of specific concentrations the for concentrations and in conditions. The effect on was calculated as the ratio of the antibody concentration after treatment with 1M NaSCN over the antibody concentration without NaSCN treatment. Note that in (B), a logarithmic scale is used for the y-axis. (C) Scatterplots of HPV16 L1 or HPV18 L1-specific log(AI) versus and respective log(antibody concentration) for individual samples from Studies 1 and 2 at Month 7 for the four age strata (N = 117). The evaluations of correlations between AIs and antibody concentrations are described by the Pearson’s r coefficients and accompanying p values.

3.3. Comparisons between AIs from two-dose (Months 0 and 6) and three-dose (Months 0, 1 and 6) vaccination schedules

The AIs of HPV16 L1- and HPV18 L1-specific antibodies and the non-vaccine strain HPV31 L1- and HPV45 L1-specific antibodies were then assessed in samples taken at Months 7, 24 and 48 from 9 to 14 year-old girls who received two vaccine doses (Months 0 and 6) and 15 to 25 year-old girls and women who received three vaccine doses (Months 0, 1 and 6). The two groups were compared, on the assumption that AIs were unaffected by age of the vaccine recipient.

At Month 7, 24 or 48, HPV16 L1- or HPV18 L1-specific GM AIs were not different between the two-dose group and the three-dose group (p > 0.385; Fig. 3A). Moreover, from Month 7 to Month 48, HPV16 L1- and HPV18 L1-specific GM AIs differed between 0.90–0.94 and 0.85–0.95, respectively, in the two-dose group; and between 0.88–0.93 and 0.81–0.89, respectively, in the three-dose group. This suggested that the relative levels of antibodies with high avidity for vaccine-specific HPV strains from Month 7 to 48 were similarly induced in the two-dose recipients to those in the three-dose recipients.

At Month 7, 24 or 48, HPV31 L1- or HPV45 L1-specific GM AIs were not different between the two-dose group and the three-dose group (p > 0.311; Fig. 3B). From Month 7 to Month 48, HPV31 L1- or HPV45 L1-specific GM AIs ranged between 0.57–0.60 and 0.56–0.70, respectively, in the two-dose group; and between 0.59–0.61 and 0.54–0.66, respectively, in the three-dose group. This suggested that the relative levels of antibodies with high avidity for non-vaccine-specific but related HPV strains were induced by age. The AI data from Study 1 and Study 2 were considered in a single statistical analysis on the assumption that there was no effect due to differences between studies.

Because no differences were detected between the HPV16 L1-specific and HPV18 L1-specific AI data sets (p = 0.982), these data were considered together in the comparisons between post-Dose 2 and post-Dose 3. In each age strata and post-Dose 3, the HPV16 L1- and HPV18 L1-specific geometric mean (GM) AIs ranged from 0.91 to 0.99 (Fig. 2), whereas post-Dose 2, the HPV16 L1- and HPV18 L1-specific GM AIs ranged from 0.58 to 0.75 (Fig. 2A). Thus at Month 7 (post-Dose 3) compared with Month 2 (post-Dose 2), the increases in the GM AIs specific for both HPV L1 antigens ranged from 1.27 to 1.56-fold (p < 0.001) in each age strata. Therefore post-Dose 3, the proportional enrichments of high-avidity antibodies, specific for either of the vaccine antigens, were detectable with these assay conditions. Moreover, post-Dose 3 compared with post-Dose 2, the HPV16 L1- and HPV18 L1-specific antibody geometric mean concentrations (GMCS) of the high avidity antibodies (antibody concentrations after NaSCN treatment) increased by 4.0–8.1-fold and 3.1–4.0-fold, respectively (p < 0.001; Fig. 2B).

The GM AIs specific for both HPV L1 antigens were not different between age strata at Month 7 and post-Dose 3 (p ≥ 0.221; 0.94–1.05-fold differences from inter-strata comparisons) even though the HPV L1-specific antibody GMCS of the high avidity antibodies differed by up to 13-fold (Fig. 2B). Therefore, the AIs at Month 7 appeared unaffected by the age of the vaccine recipient over a range of 10–55 years. Moreover, no correlations were identified between HPV16 L1 or HPV18 L1-specific AIs and the respective antibody concentrations for individual samples across the four age strata at Month 7 (Fig. 2C), suggesting that the AI measurement captures a different aspect of the antibody response to that of the antibody concentration measured by ELISA without a chaotropic agent.

Fig. 2. The evaluation of HPV-specific antibody avidities at Months 2 and 7 with a 0–1–6 month HPV16/18 vaccination schedule. Individual values (circles) and geometric means (GM; horizontal lines) with 95% confidence intervals (95%CI) of (A) HPV16 L1- and HPV18 L1-specific avidity indices and (B) HPV16 L1- and HPV18 L1-specific high-avidity antibody concentrations (measured with 1M NaSCN treatment). Samples were obtained from vaccinated 10–14 year-old girls (N = 30, post-Dose 2; N = 29, post-Dose 3) in Study 1, and 15–55 year-old girls and women, allocated to three groups stratified by age (15–28 years old [N = 35, post-Dose 2; N = 34, post-Dose 3]; 29–41 years old [N = 20, post-Dose 2, N = 21, post-Dose 3]; and 42–55 years old [N = 34, post-Dose 2 and 3]) from Study 2. The samples were taken one month post-Dose 2 (Month 2, dark grey circles) and one month post-Dose 3 (Month 7, light grey circles). The avidity index (AI) was calculated as the ratio of the antibody concentration after treatment with 1M NaSCN over the antibody concentration without NaSCN treatment. Note that in (B), a logarithmic scale is used for the y-axis. (C) Scatterplots of HPV16 L1 or HPV18 L1-specific log(AI) versus and respective log(antibody concentration) for individual samples from Studies 1 and 2 at Month 7 for the four age strata (N = 117). The evaluations of correlations between AIs and antibody concentrations are described by the Pearson’s r coefficients and accompanying p values.
similarly at each period examined (Month 7, 24 and 48) in the two-dose recipients compared with the three-dose recipients.

4. Discussion

This exploratory study supplements the observations made in the primary analysis of the HPV-16/18 vaccine clinical trial which demonstrated that the magnitude of antibody responses for the two-dose schedule (9–14 year olds) was not inferior to the three-dose schedule (15–25 year olds) [6]. Hence the limitations of the present study are that the analyses were post hoc; and, in the comparison of the two-dose versus three-dose schedules, it was assumed that the age of vaccine recipient had no effect on the magnitude of the AI. In the present study, no differences in AIs were observed at Months 7, 24 and 48 between the groups of two-dose and three-dose HPV-16/18 vaccine recipients, suggesting that the quality of the antibody responses to HPV16, 18, 31 or 45 L1 VLPs in terms of avidity was similar in the two groups. As expected, the AIs for HPV31 L1 and HPV45 L1 VLPs were relatively lower than for HPV16 and 18 L1 VLPs, since these VLPs are not vaccine types and the L1 protein sequence homologies with HPV16 and 18 L1 are 83% and 88%, respectively [27]. Therefore, and in line with what has been proposed with the heptavalent pneumococcal vaccine [28], antibody avidity, in addition to antibody concentration, can be a useful immunological attribute in the evaluation of alternative vaccine schedules.

Antigen-specific avidity has been assessed in other studies of HPV vaccines [9,10,19,20,29]. An underlying objective of the present study was to use a methodology that can easily be adopted in the clinical trial setting. Therefore, a single (1 M) concentration of the chaotropic agent NaSCN was selected and antibody concentrations, with and without chaotropic agent, were calculated from serum dilution series. Moreover, ELISA-based assays using a single concentration of chaotropic agent have been reliably used elsewhere to measure the avidity of polyclonal antibodies in human serum samples [18,30]. The one-step aspect of the assay may make it more amenable for high-throughput analyses than the two-step ELISA methodology reported by Dauner et al. [20,29]. In that methodology, antibody concentrations were measured in the first ELISA step to calculate the stipulated dilution factors for the avidity evaluation in the second ELISA step (which used a dilution series of chaotropic agent). In the present study, the selection of the 1 M concentration of NaSCN was a conservative choice to avoid potential artefacts associated with higher concentrations, such as the modification of antigen structural components (e.g. the disruption of conformational epitopes; or the instability of antigen attachment to the ELISA plate; see [29] in which Guanidine HCl and NH4SCN were evaluated).

The relevance of the avidity ELISA in this study was confirmed by detecting HPV16 and HPV18 L1-specific AI increases at post-Dose 3 (Month 7) compared with post-Dose 2 (Month 2). These increases were in line with a previous study of the same vaccine [10] and with the anticipated affinity maturation of vaccine-antigen specific antibodies [21,22]. The impact of the interval between Dose 1 and Dose 2 in the 2-dose schedule on the magnitude of the AI was not evaluated. Although the data suggested that HPV16 and HPV18 L1-specific AIs were higher one month after Dose 2 in a 0, 6 month schedule than in a 0, 1 month schedule, the length of time after Dose 1 (seven months rather than two months) may have also contributed to the magnitude of the AIs [28].

The absence of strong correlations between AIs and absolute antibody concentrations concurred with other published observations, in that the magnitude and quality of the antibody response are not temporally associated [9–11]. In one of those studies, HPV16 L1-specific AIs were only correlated with neutralisation responses at one of the several time points examined over a 36-month post-vaccination period [10]. Furthermore, although the magnitude of absolute high-avidity antibody concentrations at Month 7 appeared to vary with the age of the vaccine recipient, the AI appeared unaffected. Therefore, this suggests that antibody quality (as measured by AI) is not highly linked to antibody quantity. Instead, the magnitude of the AI may reflect the magnitude of certain aspects of the T cell response including the involvement of Th1 cells in the clonal selection of B-cell populations, such as B-memory cells and plasma cells, with high-affinity for the antigens [31]. Moreover, the induction of persistent B-memory and T cells after immunisation with HPV-16/18 vaccine has been demonstrated in several studies [11,32,33]. Hence further investigations could be conducted to identify the relationship between the avidity of HPV L1-specific antibodies, their functional activity and the induction of B-memory and T cells.

5. Conclusions

In the absence of clinical efficacy data in the 9–14 year olds, the assessment of the antibody concentration and quality in this population is crucial. Even if the clinical relevance of the avidity measurements remains to be determined, this study indicates that AIs from Month 7 to 48 appeared similar for the two-dose in 9–14 year olds and three-dose HPV-16/18 vaccine schedules in 15–25 year olds. These findings therefore complement the conclusion made in the primary analysis of the clinical trial that the two-dose schedule was immunologically non-inferior to the three-dose schedule [6]. This study also supports the use of this simple modified ELISA approach to monitor avidities for vaccine and non-vaccine specific antibodies in future HPV vaccine studies.

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Contributors
SG, LL, MB and CL developed and designed the study. LL, MB, CL and MF acquired the data. LL, MB, CL and MF performed and supervised the analysis. SG, LL, MB, CL, MF and FT were involved in the interpretation of the data. All authors were involved in the drafting of the manuscript or revising it critically for important intellectual content. All authors approved the manuscript before it was submitted by the corresponding author. All authors had full access to the data and had final responsibility to submit for publication.

Conflict of interest statement
All authors completed the ICMJE Form for disclosure of potential conflicts of interest and declared that the following interests are related to the submitted work. All authors are employees of the GlaxoSmithKline group of companies. Sandra Giannini, Clarisse Lorin and Florence Thomas report ownership of GSK stock options.

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