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Research Paper

Naturally acquired HPV antibodies against subsequent homotypic infection: A large-scale prospective cohort study

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ABSTRACT

Background: Although recent studies have suggested that naturally acquired Human papillomavirus (HPV) antibodies are partly protective against subsequent homotypic infection, the extent of protection remains indecisive. Here, we evaluate the protective effect of neutralizing and IgG antibodies simultaneously.

Methods: In a cohort of 3634 women aged 18-45 years from the control arm of a phase III trial of the HPV-16/18 bivalent vaccine, participants were tested for neutralizing antibodies by pseudovirion-based neutralization assay (PBNA) and IgG antibodies by enzyme-linked immunosorbent assay (ELISA) at baseline. HPV-16/18 incident and persistent infections were identified using cervical specimens periodically collected during the 5·5 years of follow-up. The protective effects of HPV-16/18 neutralizing and IgG antibodies against homotypic infection were assessed using a Cox proportional hazard model.

Findings: For the persistent infection (PI) endpoints of HPV-16/18 lasting for over 6/12 months, a prevalence of type-specific neutralizing antibodies was highly protective (6-month PI: hazard ratio (HR) = 0·16,

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95% confidence interval (CI): 0·04, 0·65; 12-month PI: HR = 0·23, 95% CI: 0·06, 0·94), whereas a prevalence of IgG antibodies was associated with minor and non-significant protection (6-month PI: HR = 0·66, 95% CI: 0·40, 1·09; 12-month PI: HR = 0·66, 95% CI: 0·36, 1·20). After increasing the cut-off value to the median IgG level, the risk of 6-month PI was significantly lower in seropositive vs seronegative women (HR = 0·38, 95% CI: 0·18, 0·83).

Interpretation: Naturally acquired antibodies are associated with a substantially reduced risk of subsequent homotypic infection.

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Research in context

Evidence before this study

We searched PubMed for studies published between January 1, 2000, and July 30, 2020, using the search terms "human papillomavirus" or "HPV" and "serology" or "seropositivity" or "natural immunity" or "natural infection" and examined the reference lists of eligible publications. The search was not limited to English-language publications.

Infection with human papillomavirus (HPV) may elicit an antibody response, and measurement of the antibody response is highly dependent on the assay used. Several serological assays are used to measure type-specific antibodies, of which the pseudovirion-based neutralization assay (PBNA) measures total neutralizing antibodies that functionally block the entry of pseudovirions into cultured cells, which are expected to be the primary immune mechanism for protection against HPV infection. However, this assay is highly labour-intensive and is therefore seldom used in large epidemiological studies. The virus-like particle-based enzyme-linked immunosorbent assay (VLP-ELISA), which measures a broad spectrum of IgG antibodies binding with the L1 VLP coating, including neutralizing and non-neutralizing antibodies, is most commonly used as an HPV immunity indicator. Recent studies have consistently suggested that natural antibody responses confer partial protection, especially at higher antibody titres. The majority of previous studies investigating the protection of naturally acquired antibodies used IgG as the marker, and limited work has been carried out to determine the protective effect against HPV based on neutralizing assays. Wentzensen et al and Lin et al compared the protective effect of naturally acquired antibodies obtained with different assays and suggested that the point estimates of protection for neutralizing antibodies measured by the competitive Luminex immunoassay (cLIA) or by secreted alkaline phosphatase protein neutralization assay (SEAP-NA) were stronger than those for the IgG antibodies measured by VLP-ELISA. However, these two studies were case-control studies with small sample sizes that investigated the effect of antibodies on HPV transient infection. A better quantification of the protective effect against subsequent HPV infection based on neutralizing assays requires a much larger study.

Added value of this study

Our study is the first to evaluate the natural immunity conferred with HPV neutralizing antibodies measured by PBNA in a cohort with a large sample size and long-term follow-up and to compare the protective effect with that of total IgG antibodies measured by VLP-ELISA under different cut-off settings at the same time. We found that naturally acquired antibodies are associated with a substantially reduced risk of subsequent homotypic HPV infection. Neutralizing antibodies are a more specific indicator for protective natural immunity, and binding IgG can serve as a surrogate indicator after setting a proper cut-off value.

Implications of all the available evidence

Our study shows that natural antibodies provide considerable protection against future infection. These findings provide more objective parameters for vaccine cost-effectiveness analysis and for further optimizing HPV vaccination strategies, especially designing the target population of catch-up programmes, which is of great significance for achieving the global strategic goal of eliminating cervical cancer in 2030 put forth by the WHO with a currently insufficient supply of HPV vaccines.

1. Introduction

Human papillomavirus types 16 and 18 (HPV-16 and HPV-18) infections cause approximately 70% of cases of invasive cervical cancer worldwide.[1] More than 80% of sexually active individuals will be infected with HPV during their lifetime; although the majority can clear the infection in approximately 2 years, the remaining individuals develop persistent infection that might induce cancers.[2] Host immune responses are likely to be a critical mechanism for preventing, controlling and eliminating HPV infection. It is thought that clearance of HPV infection is mainly mediated by the cellular immune response, whereas antibody responses theoretically help prevent subsequent infections.[3]

Several prophylactic HPV vaccines have been available since 2006, including HPV-16/18 bivalent vaccine (Cervarix®, GSK, and Cecolin®, Xiamen Innovax), HPV-6/11/16/18 quadrivalent vaccine (Gardasil®, Merck), and HPV-6/11/16/18/31/33/45/52/58 9-valent vaccine (Gardasil ®9, Merck). However, the current insufficient supply of the vaccines, although expected to be temporary, has greatly slowed the pace of wide implementation, especially in resource-limited areas where the burden of cervical cancer is high. It is important to develop HPV vaccination strategies based on cost-effectiveness analysis, which is of great significance for achieving the global strategic goal of eliminating cervical cancer in 2030 put forth by the WHO.[4] Mathematical modelling has been used to estimate the cost-effectiveness of HPV vaccination, which requires realistic assumptions for the transmission and infection clearance of HPV, as well as the extent and duration of acquired immunity after infection clearance.[5] Laprise JF et al[6] suggested that the results of cost-effectiveness analysis were sensitive to assumptions about natural immunity; hence, a better understanding of the extent of preventive immunity raised by natural HPV infection is crucial to accurately model the effectiveness of different vaccination strategies.

Several HPV serological assays with different properties are currently available: the pseudovirion-based neutralization assay (PBNA), which measures total neutralizing antibodies that can block the entry of HPV type-specific pseudovirions into cultured cells and are thought to be a biologically relevant subset of the antibodies; the competitive Luminex immunoassay (cLIA), designed to measure antibodies competing with a dominant type-specific monoclonal antibody against a neutralizing epitope and thus has the potential to detect the majority of the neutralizing antibodies; and the virus-like particle-based enzyme-linked immunosorbent assay (VLP-ELISA), which measures a broad spectrum of neutralizing and non-neutralizing IgG antibodies binding with the coating L1 VLP.[7] These three assays are technically different and measure different aspects of the HPV humoral immune status.

Approximately 50%-70% of individuals infected with HPV-16/18 have detectable type-specific serum antibodies.[8-12] Whether these naturally acquired antibodies protect against future infections has been debated in earlier studies,[13-15] which might be due to the heterogeneity of antibody assays, relatively small sample sizes, analytic techniques and different end points. Nevertheless, recent studies have consistently suggested that natural HPV antibodies are partly protective, especially at higher antibody titres.[16-18] However, the immunity indicator used in these studies has mostly been binding IgG antibodies measured by VLP-ELISA. The sensitivity and specificity of ELISAs are largely determined by the characteristics of coating HPV L1 antigen, which lead to high heterogenicity among different ELISAs and might cause inconsistency among different studies. Neutralizing antibodies are expected to be the primary immune mechanism for protection against HPV infection, and for most virology studies, they are thought to be the gold standard for serology investigation. PBNA theoretically measures total HPV type-specific neutralizing antibodies; however, due to the complicated experimental platform and its labour-consuming characteristics, very limited work has been done to determine the protective effect of HPV natural immunity based on neutralizing antibody assays.

Wentzensen et al[19] and Lin et al[7] compared the protective effect of natural antibodies with different assays and suggested that the point estimates of protection for neutralizing antibodies measured by cLIA or by secreted alkaline phosphatase protein neutralization assay (SEAP-NA) were stronger than those for IgG antibodies measured by VLP-ELISA. However, these two studies were case-control studies with small sample sizes (N = 933 and N = 388, respectively) and investigated the effect of antibodies on HPV transient infection. A better quantification of the protection against subsequent HPV infection based on neutralizing assay requires a much larger study. Here, we evaluate the protection against HPV-16/18 infection of naturally acquired antibodies in a 66-month follow-up period based on the PBNA assay in a cohort of women from the control arm of a large multicentre phase III trial of the novel *Escherichia coli*-based recombinant HPV-16 and -18 bivalent vaccine (Cecolin®), expected to determine the protective extent of natural HPV antibodies. As VLP-ELISA is a much easier platform to set up than PBNA, we compared the protective effect between neutralizing antibodies and that of total IgG antibodies measured by ELISA and explored the potential for IgG antibodies to act as a surrogate for neutralizing antibodies to investigate the protective effect of naturally induced antibodies.

2. Methods

2.1. Study population and procedures

Study participants were women randomized to the control arm of the multicentre, randomized, double-blind, controlled phase III trial of the HPV-16/18 bivalent vaccine Cecolin® (ClinicalTrials.gov, NCT01735006). The enrolled participants were healthy women aged 18 to 45 years old who were not pregnant, were immunocompetent, and had 1-4 sexual partners. The methodologies, inclusion and exclusion criteria have been previously described in detail.[20] Our analysis included women DNA negative for HPV-16 and/or HPV-18, with no high-grade cervical intraepithelial lesion or cancer (CIN2+) at baseline, who had received at least one control vaccine dose and had at least one effective follow-up visit (Figure 1).

Serum samples of all participants were collected at day 0 before vaccination. Gynaecological examinations were performed at day 0 and months 7, 12, 18, 24, 30, 42, 54, and 66. At these visits, endocervical swab samples were collected for Papanicolaou testing and HPV DNA typing. Cytology results were reported according to the Bethesda system-2001. Women with abnormal cytological test results, excluding atypical squamous cells of undetermined significance (ASC-US)/high-risk HPV-negative, were referred for colposcopy and biopsied if necessary according to the colposcopy management algorithm. Paraffin-embedded biopsied tissue specimens diagnosed as cervical intraepithelial neoplasia grade 1 or more severe (CIN1+) were also typed for HPV DNA.[20] Written informed consent was obtained from all participants, and the protocol was approved by independent ethics committees.

2.2. Antibody detection

Anti-HPV-16 and -18 of baseline serum samples were tested by both PBNA and ELISA. Detailed procedures have been described previously.[20-22] In brief, for PBNA, the serum samples were serially diluted starting at 1:20 in 2-fold increments and then mixed with HPV pseudovirions, after which the mixtures were transferred to 293FT cell monolayers and incubated at 37°C and 5% CO_2. A positive sample was defined as one that caused a 50% reduction or more in green fluorescent protein expression (GFP) compared with the negative control, and the neutralization titres were defined as the highest dilution of positive samples. The cut-off titres of PBNA were set as 1:20. For ELISA, each well of a 96-well microtitre plate was coated with HPV L1 VLPs expressed by *E. coli*, and then serially diluted serum samples were added. The optical density was read at 450/620 nm. The positive samples were quantified using standard curves of references traceable to the World Health Organization international standards for antibodies against HPV-16 (NIBSC code 05/134) or HPV-18 (NIBSC code 10/140) expressed in international units (IUs), and the cut-off values were 3·0 IU/ml for HPV-16 and 2·1 IU/ml for HPV-18.

2.3. HPV DNA detection

HPV DNA testing was performed using the HPV DNA enzyme immunoassay (DEIA) (Labo Biomedical Products, the Netherlands). Samples with positive findings were further typed by broad spectrum PCR SPF10-LiPA 25 (Version 1) (Labo Biomedical Products) for 13 oncogenic HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) and 12 non-oncogenic HPV types (6, 11, 34, 40, 42, 43, 44, 53, 54, 66, 70, and 74) and by HPV-16/18-specific polymerase chain reactions (HPV TS16/18, Labo Biomedical Products). A positive result for HPV-16 or HPV-18 was defined as the presence of the relevant type of HPV DNA by either LiPA or HPV TS16/18.

2.4. Statistical analysis

2.4.1. Exposure variables

The main exposure variables were HPV-16 and -18 serostatus, which were expressed as binary variables (seropositive or seronegative) at enrolment. To increase the statistical power of the analysis, we also combined the protective effects of natural antibod-

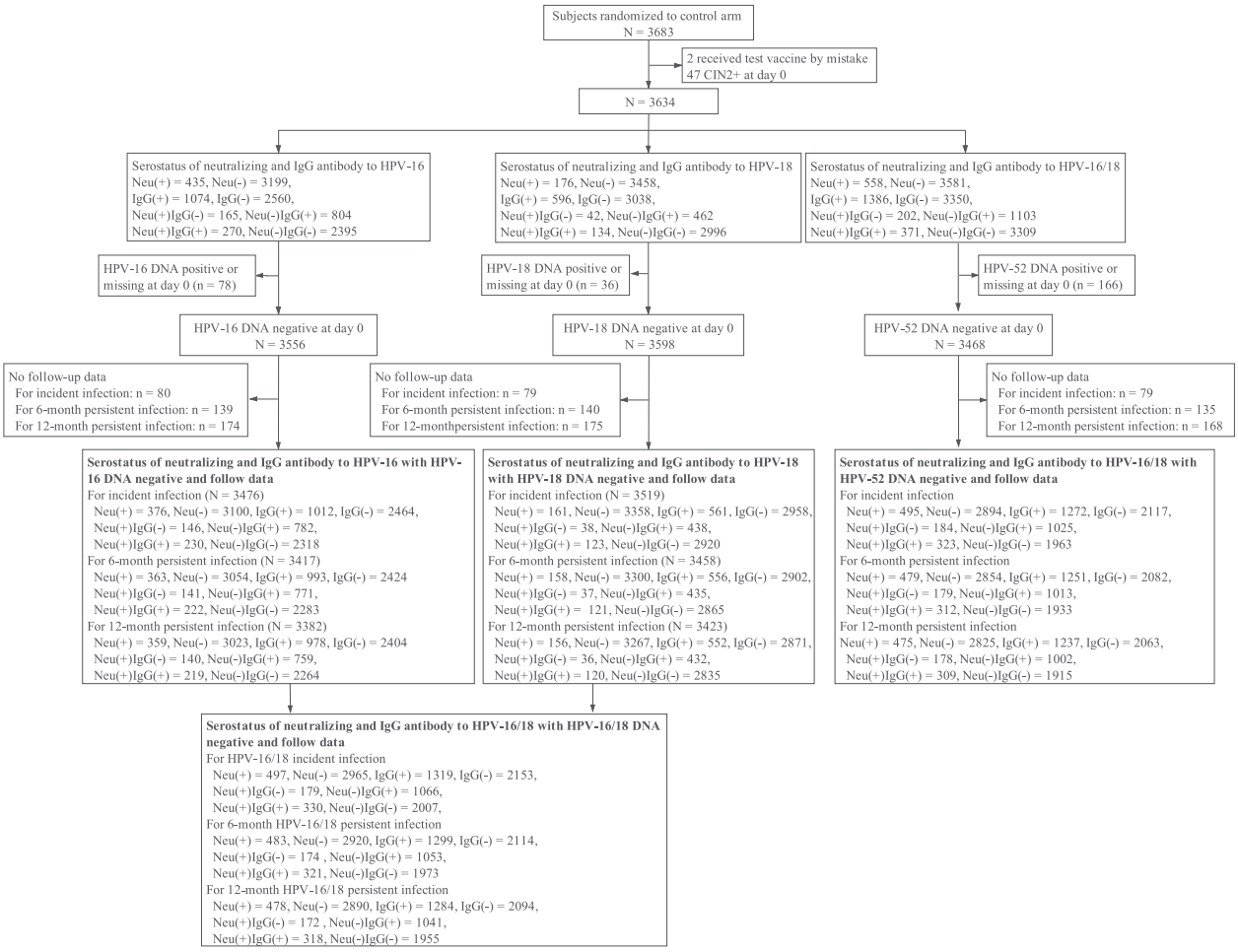

Figure 1. Flow chart of the participants. CIN2+: High-grade cervical intraepithelial lesion or cancer; Neu (+): Seropositive for neutralizing antibodies; Neu (-): Seronegative for neutralizing antibodies; IgG (+): Seropositive for IgG antibodies; IgG (-): Seronegative for IgG antibodies; Neu (-) IgG (-): Seronegative for both neutralizing and IgG antibodies; Neu (-) IgG (+): Seronegative for neutralizing antibodies but seropositive for IgG antibodies; Neu (+) IgG (-): Seropositive for neutralizing antibodies but seronegative for IgG antibodies; Neu (+) IgG (+): Seropositive for both neutralizing and IgG antibodies;

ies against future homotypic infections with HPV-16 and HPV-18. According to the serostatus of neutralizing and IgG antibodies to HPV-16 and HPV-18, women were classified as three analysis set groups: 1) seronegative for both HPV-16 and HPV-18 neutralizing antibodies (HPV-16/18 Neu (-)); seropositive for HPV-16 and/or HPV-18 neutralizing antibodies (HPV-16/18 Neu (+)); 2) seronegative for both HPV-16 and HPV-18 IgG antibodies (HPV-16/18 IgG (-)); seropositive for HPV-16 and/or HPV-18 IgG antibodies (HPV-16/18 IgG (+)); 3) seronegative for both HPV-16 neutralizing and IgG antibodies and seronegative for both HPV-18 neutralizing and IgG antibodies (HPV-16/18 Neu (-) IgG (-)); seronegative for HPV-16 neutralizing antibodies but seropositive for HPV-16 IgG antibodies and/or seronegative for HPV-18 neutralizing antibodies but seropositive for HPV-18 IgG antibodies (HPV-16/18 Neu (-) IgG (+)); seropositive for HPV-16 neutralizing antibodies but seronegative for HPV-16 IgG antibodies and/or seropositive for HPV-18 neutralizing antibodies but seronegative for HPV-18 IgG antibodies (HPV-16/18 Neu (+) IgG (-)); seropositive for both HPV-16 neutralizing and IgG antibodies and/or seropositive for both HPV-18 neutralizing and IgG antibodies (HPV-16/18 Neu (+) IgG (+)) (Table 1).

2.4.2. Outcome variables

Endpoints evaluated were newly detected infection, 6/12-month persistent infection (6-m PI and 12-m PI), separately defined as (1) incident infection: detection of the specific-type HPV DNA at least once during the follow-up period; (2) 6/12 m PI: detection of the same HPV type in at least 2 samples not interrupted by negative samples over a minimum of 150 days (6-m PI) and of 300 days (12-m PI).

2.4.3. Statistical methods

Incidence was calculated as the number of detected events divided by the total person-time. Person-time was calculated as the sum of the follow-up for each participant expressed in years. The follow-up period started on the day after the first vaccination and ended on the date of the first occurrence of the type-specific endpoint or the date of the last effective gynaecological visit. The analysis unit for grouped infection was based on the individual, and infection that occurred in the relative baseline serostatus cohort of the corresponding type was considered an endpoint event (Table 1). The relationship between exposure and risk of newly detected infections was assessed using a Cox proportional hazard model. The Kaplan-Meier method was used to construct the cumulative incidence of infections by serostatus group. Log-rank tests were used to compare the differences in the cumulative incidence across groups. All statistical analyses were conducted using SAS version 9.4 software (SAS Institute, Cary, North Carolina).

2.4.4. Role of the funding source

The funder of the study had no role in the study design, data collection, data analysis, data interpretation, or writing of the re-

Table 1
Categorization scheme of exposure.

Exposure	HPV-16 serostatus Neu	IgG	HPV-18 serostatus Neu	IgG	The endpoint type of observation
HPV-16/18 Neu (-)	-	NA	-	NA	HPV-16 and HPV-18
HPV-16/18 Neu (+)	+	NA	-	NA	HPV-16
	-	NA	+	NA	HPV-18
	+	NA	+	NA	HPV-16 and HPV-18
HPV-16/18 IgG (-)	NA	-	NA	-	HPV-16 and HPV-18
HPV-16/18 IgG (+)	NA	+	NA	-	HPV-16
	NA	-	NA	+	HPV-18
	NA	+	NA	+	HPV-16 and HPV-18
HPV-16/18 Neu (-) IgG (-)	-	-	-	-	HPV-16 and HPV-18
HPV-16/18 Neu (-) IgG (+)	-	+	-	-	HPV-16
	-	+	+	-	HPV-16
	-	+	+	+	HPV-16
	-	-	-	+	HPV-18
	+	-	-	+	HPV-18
	+	+	-	+	HPV-18
	-	+	-	+	HPV-16 and HPV-18
HPV-16/18 Neu (+) IgG (-)	+	-	-	-	HPV-16
	+	-	-	+	HPV-16
	+	-	+	+	HPV-16
	+	-	+	-	HPV-16 and HPV-18
	-	-	+	-	HPV-18
	-	+	+	-	HPV-18
	+	+	+	-	HPV-18
HPV-16/18 Neu (+) IgG (+)	+	+	-	-	HPV-16
	+	+	-	+	HPV-16
	+	+	+	-	HPV-16
	+	+	+	+	HPV-16 and HPV-18
	-	-	+	+	HPV-18
	-	+	+	+	HPV-18
	+	-	+	+	HPV-18

Neu: neutralizing antibodies, IgG: IgG antibodies; +: seropositive; -: seronegative. NA: not applicable, meaning regardless of the serostatus.

port. The corresponding authors had full access to all of the data, as well as the final responsibility to submit for publication.

3. Results

A total of 3634 participants were included in the study (Figure 1). The seroprevalences of HPV-16 neutralizing antibodies and IgG antibodies were 12·0% (435 of 3634) and 29·6% (1074 of 3634), respectively. For HPV-18, the seroprevalences of neutralizing antibodies and IgG antibodies were 4·8% (176 of 3634) and 16·4% (596 of 3634), respectively. Baseline characteristics among women with different serostatuses are shown in Table 2 and Tables S1-2 in the Supplementary Tables. The mean age of the participants was approximately 30 years and was similar in different serostatus groups. At baseline, compared with women who were negative for both neutralizing and IgG antibodies, women with neutralizing antibodies more frequently had abnormal cytological findings (ASC-US+) (11·5% vs 7·1%, *P*=0·0011), while the prevalence was the same in women with IgG antibodies (8·4% vs 7·1%, *P*=0·1515).

The observed protective effects of neutralizing and IgG antibodies against subsequent infection were very similar in univariate and age-adjusted analyses (Table 3). In age-adjusted analyses, both neutralizing antibodies and IgG antibodies were associated with protection against subsequent HPV-16/18 incident infections. Although the point estimate of the neutralizing antibodies showed stronger protection, the difference was not significant (Neu: HR=0·41, 95% CI, 0·25, 0·67; IgG: HR=0·60, 95% CI, 0·46, 0·79). We observed that neutralizing antibodies significantly lowered the risk of subsequent homotypic HPV-16/18 persistent infection (6-m PI: HR = 0·16, 95% CI: 0·04, 0·65; 12-m PI: HR = 0·23, 95% CI: 0·06, 0·94) but that the presence of IgG antibodies modestly and non-significantly lowered the risk of subsequent homotypic HPV-16/18 persistent infection (6-m PI: HR = 0·66, 95% CI: 0·40, 1·09; 12-m PI: HR = 0·66, 95% CI: 0·36, 1·20). Moreover, concomitant positivity for IgG antibodies did not significantly increase the preventive effects of neutralizing antibodies (Table 3). The Kaplan-Meier plots showed that the preventive effects of neutralizing antibodies did not decline with time for at least 5 years (Figure 2).

The protective effect of binding IgG antibodies based on increased cut-off values determined from quartile IgG antibody levels of seropositive participants was also analysed. After increasing the cut-off value, positive IgG status showed stronger protection efficacy against subsequent HPV infection. With setting the cut-off to the median level (6·6 IU/ml for HPV-16 and 4·0 IU/ml for HPV-18), the risk of HPV-16/18 incident infection and 6-month PI was statistically lower in seropositive vs seronegative women (incident infection: HR = 0·54, 95% CI: 0·38, 0·78; 6-m PI: HR = 0·38, 95% CI: 0·18, 0·83). The point estimate protective effect of IgG peaked at the cut-off setting of the median IgG level, which was still lower than that of PBNA neutralizing antibodies, and the calculated protection efficacy was not further increased by increasing the cut-off value to the 75% quantile (Q3) (Table 4). When analysing the different combined IgG and neutralizing antibody statuses, moderate protection against 6-month persistent infection was observed in HPV-16/18 Neu (-) IgG (+) compared with HPV-16/18 Neu (-) IgG (-); however, the point estimate was also weaker than HPV-16/18 Neu (+) IgG (-) (HR = 0·43, 95% CI: 0·19, 1·00 vs HR = 0·13, 95% CI: 0·02, 0·95) (Table S3 in the Supplementary Tables).

When natural immunity to HPV-16 and HPV-18 was analysed independently, limited to the sample size and low incidence, the data did not show a significantly reduced risk of infection by HPV-16 or HPV-18. However, similar trends were observed for the preventive effects of neutralizing and IgG antibodies (Table 5, Tables S4-6 in the Supplementary Tables, and Figures S1-2 in the Supplementary Figures).

Table 2
Baseline characteristics of the participants in different HPV-16/18 serostatus groups.

	Neutralizing antibodies		Binding antibodies		Combination of neutralizing and binding antibodies			
	Neu (-)	Neu (+)	IgG (-)	IgG (+)	Neu (-) IgG (-)	Neu (-) IgG (+)	Neu (+) IgG (-)	Neu (+) IgG (+)
Number of participants	3076	558	2248	1386	2082	1103	202	371
Mean age ± SD (y)	29·9 ± 7·4	29·9 ± 7·2	30·0 ± 7·4	29·7 ± 7·2	30·0± 7·4	29·8 ± 7·2	30·4 ± 7·5	29·8 ± 7·1
Cytological findings at day 0, n (%) [#]								
Normal (NILM)	2859 (93·0)	493 (88·4)	2085 (92·8)	1267 (91·4)	1933 (92·8)	1020 (92·5)	183 (90·6)	323 (87·1)
Abnormal (ASC-US+)	215 (7·0)	64 (11·5)	162 (7·2)	117 (8·4)	148 (7·1)	82 (7·4)	19 (9·4)	47 (12·7)
ASC-US	138 (4·5)	45 (8·1)	111 (4·9)	72 (5·2)	101 (4·9)	47 (4·3)	13 (6·4)	33 (8·9)
HC2 (-)	88 (2·9)	21 (3·8)	72 (3·2)	37 (2·7)	68 (3·3)	24 (2·2)	5 (2·5)	16 (4·3)
HC2 (+)	50 (1·6)	24 (4·3)	39 (1·7)	35 (2·5)	33 (1·6)	23 (2·1)	8 (4·0)	17 (4·6)
LSIL	69 (2·2)	14 (2·5)	45 (2·0)	38 (2·7)	42 (2·0)	30 (2·7)	4 (2·0)	11 (3·0)
HSIL	1 (0.0)	4 (0·7)	2 (0·1)	3 (0·2)	1 (0·1)	1 (0·1)	1 (0·5)	3 (0·8)
ASC-H	5 (0·2)	1 (0·2)	3 (0·1)	3 (0·2)	3 (0·1)	3 (0·3)	1 (0·5)	0 (0)
AIS/AGC	2 (0·1)	0 (0)	1 (0·0)	1 (0·1)	1 (0·1)	1 (0·1)	0 (0)	0 (0)
Unsatisfactory	2 (0·1)	1 (0·2)	1 (0·0)	2 (0·1)	1 (0·1)	1 (0·1)	0 (0)	1 (0·3)

Neu (-): Seronegative for both HPV-16 and HPV-18 neutralizing antibodies; Neu (+): Seropositive for HPV-16 and/or HPV-18 neutralizing antibodies; IgG (-): Seronegative for both HPV-16 and HPV-18 IgG antibodies; IgG (+): Seropositive for HPV-16 and/or HPV-18 IgG antibodies; Neu (-) IgG (-): Seronegative for both HPV-16 neutralizing and IgG antibodies and seronegative for both HPV-18 neutralizing and IgG antibodies; Neu (-) IgG (+): Seronegative for HPV-16 neutralizing antibodies but seropositive for HPV-16 IgG antibodies and/or seronegative for HPV-18 neutralizing antibodies but seropositive for HPV-18 IgG antibodies; Neu (+) IgG (-): Seropositive for HPV-16 neutralizing antibodies but seronegative for HPV-16 IgG antibodies and/or seropositive for HPV-18 neutralizing antibodies but seronegative for HPV-18 IgG antibodies; Neu (+) IgG (+): Seropositive for both HPV-16 neutralizing and IgG antibodies and/or seropositive for both HPV-18 neutralizing and IgG antibodies

[#] NILM: negative for intraepithelial lesion or malignancy; ASC-US: Atypical squamous cells of undetermined significance; LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesion; ASC-H: atypical squamous cells cannot exclude high-grade lesion; AIS: adenocarcinoma in situ; AGC: atypical glandular cells; HC2 (-): negative on the Hybrid Capture-2 test; HC2 (+): positive on the Hybrid Capture-2 test.

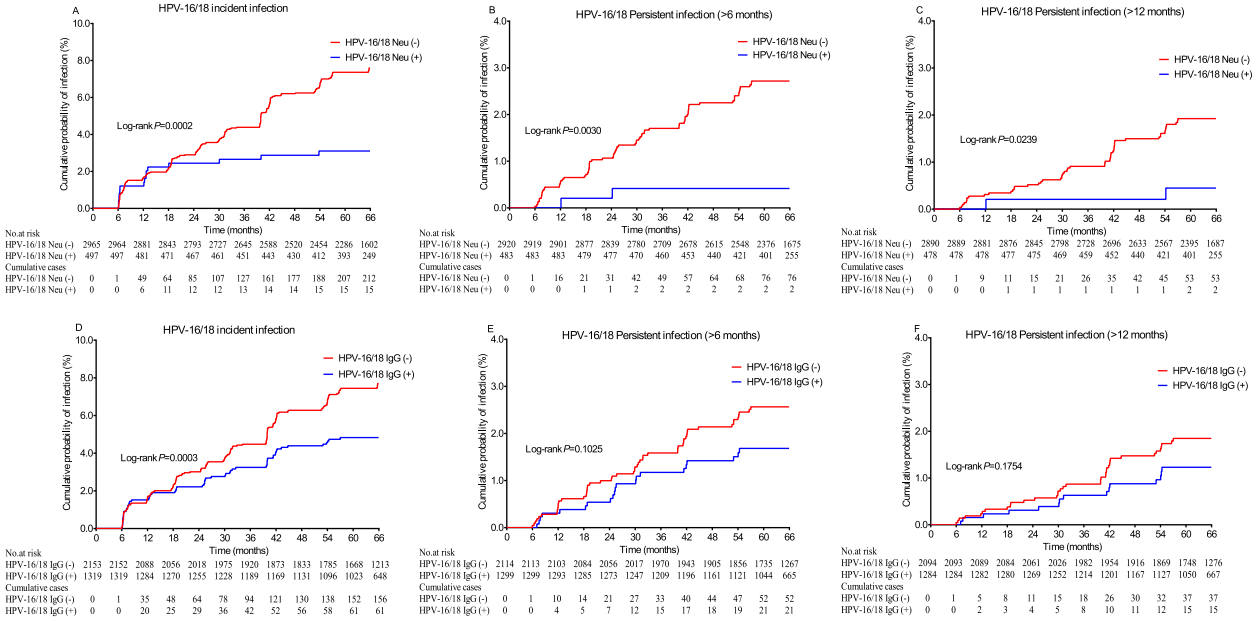

Figure 2. Kaplan-Meier estimates of the cumulative incidence of HPV-16/18 incident infection (A, D), persistent infection (>6 months) (B, E) and persistent infection (>12 months) (C, F) among different HPV-16/18 serostatus groups. The log-rank test was used to analyse the differences among different serostatuses. HPV-16/18 Neu (-): seronegative for both HPV-16 and HPV-18 neutralizing antibodies; HPV-16/18 Neu (+): seropositive for HPV-16 and/or HPV-18 neutralizing antibodies; HPV-16/18 IgG (-): seronegative for both HPV-16 and HPV-18 IgG antibodies; HPV-16/18 IgG (+): seropositive for HPV-16 and/or HPV-18 IgG antibodies.

4. Discussion

In this study, we observed an association between HPV-16/18 seropositivity and protection from subsequent infection over 5·5 years of follow-up, though the extent of protection was dependent on the serological indicators used. Naturally acquired HPV-16/18 neutralizing antibodies significantly reduced the risk of subsequent 6-month infection by 84%, while the binding IgG antibodies modestly and non-significantly lowered the risk by 34% during the 66-month follow-up period. After increasing the cut-off value of IgG antibodies to the median IgG antibody level of seropositive participants, IgG antibodies also showed a significant protective effect against future infection, albeit with a relatively lower point estimate (62%) than those of neutralizing antibodies.

The strength of our study is its first assessment of natural immunity conferred with neutralizing antibodies measured by PBNA in a cohort with a large sample size and long-term follow-up, and the laboratory measurements were fully validated to support the licensure of a new vaccine.[20] At the same time, we compared the protective effect of total IgG antibodies measured by VLP-ELISA under different cut-off settings with that of PBNA neutralizing antibody.

Previous studies suggested that the risk of subsequent HPV infection showed a decreasing trend with increasing homotypic HPV-specific IgG titres.[16]· [18] Our study also showed that the protective effect of IgG antibodies was mild at the original cut-off value but that it increased after enhancing the IgG cut-off value set. After increasing the cut-off to the median IgG level of seropositive par-

Table 3
The risk of newly detected HPV-16/18 infection according to the HPV-16/18 serostatus.

HPV-16/18 serostatus*	No. of participants	Person-years	No. of events	Incidence (95% CI), per 100 person-years	Hazard ratio (95% CI)	*P* Value	Adjusted Hazard ratio (95% CI) #	*P* Value #
Endpoint: HPV-16/18 incident infection								
Neu (-)	2965	14,970	253	1·69 (1·49, 1·91)	1·00	••	1·00	••
Neu (+)	497	2525	17	0·67 (0·42, 1·08)	0·40 (0·25, 0·66)	0·0003	0·41 (0·25, 0·67)	0·0003
IgG (-)	2153	10,875	190	1·75 (1·52, 2·01)	1·00	••	1·00	••
IgG (+)	1319	6691	70	1·05 (0·83, 1·32)	0·60 (0·46, 0·80)	0·0003	0·60 (0·46, 0·79)	0·0003
Neu (-) IgG (-)	2007	10,164	179	1·76 (1·52, 2·04)	1·00	••	1·00	••
Neu (-) IgG (+)	1066	5409	58	1·07 (0·83, 1·39)	0·62 (0·46, 0·83)	0·0014	0·61 (0·46, 0·82)	0·0011
Neu (+) IgG (-)	179	902	5	0·55 (0·23, 1·33)	0·33 (0·14, 0·80)	0·0146	0·34 (0·14, 0·81)	0·0158
Neu (+) IgG (+)	330	1687	12	0·71 (0·40, 1·25)	0·41 (0·23, 0·73)	0·0024	0·41 (0·23, 0·73)	0·0025
Endpoint: 6-month persistent HPV-16/18 infection								
Neu (-)	2920	15,283	76	0·50 (0·40, 0·62)	1·00	••	1·00	••
Neu (+)	483	2559	2	0·08 (0·02, 0·31)	0·16 (0·04, 0·64)	0·0097	0·16 (0·04, 0·65)	0·0102
IgG (-)	2114	11,112	52	0·47 (0·36, 0·61)	1·00	••	1·00	••
IgG (+)	1299	6784	21	0·31 (0·20, 0·47)	0·66 (0·40, 1·09)	0·1052	0·66 (0·40, 1·09)	0·1022
Neu (-) IgG (-)	1973	10,378	50	0·48 (0·37, 0·64)	1·00	••	1·00	••
Neu (-) IgG (+)	1053	5470	20	0·37 (0·24, 0·57)	0·75 (0·45, 1·27)	0·2852	0·75 (0·45, 1·26)	0·2784
Neu (+) IgG (-)	174	913	1	0·11 (0·02, 0·78)	0·23 (0·03, 1·64)	0·1406	0·23 (0·03, 1·65)	0·1435
Neu (+) IgG (+)	321	1711	1	0·06 (0·01, 0·41)	0·12 (0·02, 0·88)	0·0364	0·12 (0·02, 0·88)	0·0367
Endpoint: 12-month persistent HPV-16/18 infection								
Neu (-)	2890	15,320	53	0·35 (0·26, 0·45)	1·00	••	1·00	••
Neu (+)	478	2550	2	0·08 (0·02, 0·31)	0·23 (0·06, 0·93)	0·0390	0·23 (0·06, 0·94)	0·0408
IgG (-)	2094	11,135	37	0·33 (0·24, 0·46)	1·00	••	1·00	••
IgG (+)	1284	6783	15	0·22 (0·13, 0·37)	0·66 (0·36, 1·21)	0·1786	0·66 (0·36, 1·20)	0·1756
Neu (-) IgG (-)	1955	10,398	36	0·35 (0·25, 0·48)	1·00	••	1·00	••
Neu (-) IgG (+)	1041	5475	14	0·26 (0·15, 0·43)	0·74 (0·40, 1·36)	0·3277	0·73 (0·40, 1·36)	0·3222
Neu (+) IgG (-)	172	910	1	0·11 (0·02, 0·78)	0·32 (0·04, 2·30)	0·2552	0·32 (0·04, 2·32)	0·2592
Neu (+) IgG (+)	318	1704	1	0·06 (0·01, 0·42)	0·17 (0·02, 1·23)	0·0793	0·17 (0·02, 1·24)	0·0798

* Neu (-): Seronegative for both HPV-16 and HPV-18 neutralizing antibodies; Neu (+): Seropositive for HPV-16 and/or HPV-18 neutralizing antibodies; IgG (-): Seronegative for both HPV-16 and HPV-18 IgG antibodies; IgG (+): Seropositive for HPV-16 and/or HPV-18 IgG antibodies; Neu (-) IgG (-): Seronegative for both HPV-16 neutralizing and IgG antibodies and seronegative for both HPV-18 neutralizing and IgG antibodies; Neu (-) IgG (+): Seronegative for HPV-16 neutralizing antibodies but seropositive for HPV-16 IgG antibodies and/or seronegative for HPV-18 neutralizing antibodies but seropositive for HPV-18 IgG antibodies; Neu (+) IgG (-): Seropositive for HPV-16 neutralizing antibodies but seronegative for HPV-16 IgG antibodies and/or seropositive for HPV-18 neutralizing antibodies but seronegative for HPV-18 IgG antibodies; Neu (+) IgG (+): Seropositive for both HPV-16 neutralizing and IgG antibodies and/or seropositive for both HPV-18 neutralizing and IgG antibodies.

Adjusted for continuous age at enrolment.

Table 4
The risk of newly detected HPV-16/18 infection according to HPV-16/18 IgG serostatus depended on different cut-off values.

HPV-16/18 serostatus*	No. of participants	Person-years	No. of events	Incidence (95% CI), per 100 person-years	Hazard ratio (95% CI)	*P* Value	Adjusted Hazard ratio (95% CI) #	*P* Value#
Endpoint: HPV-16/18 incident infection								
Cut-off (Q1): HPV-16: 4·4; HPV-18: 2·8								
IgG (-)	2394	12,090	205	1·70 (1·48, 1·94)	1·00	••	1·00	••
IgG (+)	1072	5441	56	1·03 (0·79, 1·34)	0·61 (0·46, 0·82)	0·0011	0·61 (0·45, 0·82)	0·0010
Cut-off (Q2): HPV-16: 6·6; HPV-18: 4·0								
IgG (-)	2751	13,855	233	1·68 (1·48, 1·91)	1·00	••	1·00	••
IgG (+)	711	3639	33	0·91 (0·64, 1·28)	0·54 (0·38, 0·78)	0·0009	0·54 (0·38, 0·78)	0·0009
Cut-off (Q3): HPV-16: 10·1; HPV-18: 7·6								
IgG (-)	3084	15,548	260	1·67 (1·48, 1·89)	1·00	••	1·00	••
IgG (+)	374	1886	19	1·01 (0·64, 1·58)	0·60 (0·38, 0·95)	0·0300	0·61 (0·38, 0·96)	0·0344
Endpoint: 6-month persistent infection								
Cut-off (Q1): HPV-16: 4·4; HPV-18: 2·8								
IgG (-)	2352	12,338	59	0·48 (0·37, 0·62)	1·00	••	1·00	••
IgG (+)	1055	5527	16	0·29 (0·18, 0·47)	0·60 (0·35, 1·05)	0·0725	0·60 (0·35, 1·04)	0·0708
Cut-off (Q2): HPV-16: 6·6; HPV-18: 4·0								
IgG (-)	2702	14,143	70	0·49 (0·39, 0·63)	1·00	••	1·00	••
IgG (+)	701	3705	7	0·19 (0·09, 0·40)	0·38 (0·18, 0·83)	0·0149	0·38 (0·18, 0·83)	0·0154
Cut-off (Q3): HPV-16: 10·1; HPV-18: 7·6								
IgG (-)	3032	15,876	79	0·50 (0·40, 0·62)	1·00	••	1·00	••
IgG (+)	367	1925	4	0·21 (0·08, 0·55)	0·42 (0·15, 1·14)	0·0875	0·42 (0·15, 1·15)	0·0921
Endpoint: 12-month persistent infection								
Cut-off (Q1): HPV-16: 4·4; HPV-18: 2·8								
IgG (-)	2328	12,365	41	0·33 (0·24, 0·45)	1·00	••	1·00	••
IgG (+)	1044	5523	13	0·24 (0·14, 0·41)	0·71 (0·38, 1·32)	0·2754	0·71 (0·38, 1·32)	0·2714
Cut-off (Q2): HPV-16: 6·6; HPV-18: 4·0								
IgG (-)	2675	14,184	47	0·33 (0·25, 0·44)	1·00	••	1·00	••
IgG (+)	693	3696	6	0·16 (0·07, 0·36)	0·49 (0·21, 1·14)	0·0977	0·49 (0·21, 1·15)	0·1006
Cut-off (Q3): HPV-16: 10·1; HPV-18: 7·6								
IgG (-)	3002	15,919	54	0·34 (0·26, 0·44)	1·00	••	1·00	••
IgG (+)	362	1921	4	0·21 (0·08, 0·55)	0·61 (0·22, 1·69)	0·3429	0·62 (0·23, 1·72)	0·3580

* IgG (-): Seronegative for both HPV-16 and HPV-18 IgG antibodies; IgG (+): Seropositive for HPV-16 and/or HPV-18 IgG antibodies;
# Adjusted for continuous age at enrolment.

Table 5
The risk of newly detected HPV-16 infection according to HPV-16 serostatus.

HPV-16 serostatus*	No. of subjects	Person-years	No. of events	HPV-16 incidence (95%CI) per 100 person-years	Hazard ratio (95%CI)	P Value	Adjusted Hazard ratio (95% CI) #	P Value #
Endpoint: incident infection								
Neu (-)	3100	15,855	178	1·12 (0·97, 1·30)	1·00	••	1·00	••
Neu (+)	376	1888	15	0·79 (0·48, 1·32)	0·72 (0·43, 1·23)	0·2276	0·73 (0·43, 1·24)	0·2400
IgG (-)	2464	12,612	140	1·11 (0·94, 1·31)	1·00	••	1·00	••
IgG (+)	1012	5131	53	1·03 (0·79, 1·35)	0·93 (0·68, 1·28)	0·6734	0·93 (0·68, 1·27)	0·6469
Neu (-) IgG (-)	2318	11,880	135	1·14 (0·96, 1·35)	1·00	••	1·00	••
Neu (-) IgG (+)	782	3975	43	1·08 (0·80, 1·46)	0·96 (0·68, 1·35)	0·8056	0·95 (0·67, 1·34)	0·7729
Neu (+) IgG (-)	146	732	5	0·68 (0·28, 1·64)	0·63 (0·26, 1·54)	0·3087	0·64 (0·26, 1·56)	0·3222
Neu (+) IgG (+)	230	1155	10	0·87 (0·47, 1·61)	0·77 (0·40, 1·46)	0·4190	0·77 (0·41, 1·46)	0·4242
Endpoint: 6-month persistent infection								
Neu (-)	3054	16,004	59	0·37 (0·29, 0·48)	1·00	••	1·00	••
Neu (+)	363	1920	2	0·10 (0·03, 0·42)	0·28 (0·07, 1·15)	0·0781	0·28 (0·07, 1·16)	0·0801
IgG (-)	2424	12,733	47	0·37 (0·28, 0·49)	1·00	••	1·00	••
IgG (+)	993	5190	14	0·27 (0·16, 0·46)	0·73 (0·40, 1·32)	0·2966	0·73 (0·40, 1·32)	0·2908
Neu (-) IgG (-)	2283	11,991	46	0·38 (0·29, 0·51)	1·00	••	1·00	••
Neu (-) IgG (+)	771	4013	13	0·32 (0·19, 0·56)	0·84 (0·45, 1·56)	0·5793	0·84 (0·45, 1·55)	0·5681
Neu (+) IgG (-)	141	742	1	0·13 (0·02, 0·96)	0·35 (0·05, 2·53)	0·2976	0·35 (0·05, 2·56)	0·3030
Neu (+) IgG (+)	222	1177	1	0·08 (0·01, 0·60)	0·22 (0·03, 1·60)	0·1350	0·22 (0·03, 1·61)	0·1362
Endpoint: 12-month persistent infection								
Neu (-)	3023	16,021	40	0·25 (0·18, 0·34)	1·00	••	1·00	••
Neu (+)	359	1912	2	0·10 (0·03, 0·42)	0·42 (0·10, 1·73)	0·2287	0·42 (0·10, 1·73)	0·2301
IgG (-)	2404	12,753	32	0·25 (0·18, 0·35)	1·00	••	1·00	••
IgG (+)	978	5180	10	0·19 (0·10, 0·36)	0·77 (0·38, 1·56)	0·4634	0·77 (0·38, 1·56)	0·4615
Neu (-) IgG (-)	2264	12,012	31	0·26 (0·18, 0·37)	1·00	••	1·00	••
Neu (-) IgG (+)	759	4009	9	0·22 (0·12, 0·43)	0·87 (0·41, 1·82)	0·7044	0·87 (0·41, 1·82)	0·7017
Neu (+) IgG (-)	140	741	1	0·13 (0·02, 0·96)	0·52 (0·07, 3·81)	0·5201	0·52 (0·07, 3·83)	0·5224
Neu (+) IgG (+)	219	1171	1	0·09 (0·01, 0·61)	0·33 (0·05, 2·42)	0·2756	0·33 (0·05, 2·42)	0·2762

* Neu (-): Seronegative for HPV-16 neutralizing antibodies; Neu (+): Seropositive for HPV-16 neutralizing antibodies; IgG (-): Seronegative for HPV-16 IgG antibodies; IgG (+): Seropositive for HPV-16 IgG antibodies; Neu (-) IgG (-): Seronegative for both HPV-16 neutralizing and IgG antibodies; Neu (-) IgG (+): Seronegative for HPV-16 neutralizing antibodies but seropositive for HPV-16 IgG antibodies; Neu (+) IgG (-): Seropositive for HPV-16 neutralizing antibodies but seronegative for HPV-16 IgG antibodies; Neu (+) IgG (+): Seropositive for both HPV-16 neutralizing and IgG antibodies;

Adjusted for continuous age at enrolment.

ticipants, positive IgG status also showed higher protection efficacy against subsequent HPV infection, and the protective effect could not be further increased by increasing the cut-off value to the 75% quartile. Nonetheless, the point estimate of the protective effect of positive IgG (at its optimal cut-off value) remained slightly lower than that of neutralizing antibodies (HR: 0·38 vs 0·16 for the 6-m PI endpoint) (Table 3 and Table 4). The data imply that neutralizing antibodies are a more specific indicator for protective natural immunity assays and that binding IgG can serve as a surrogate indicator after setting a proper cut-off value.

Based on the original cut-off (3·0 IU/ml for HPV-16 and 2·1 IU/ml for HPV-18) of our ELISA, the reported seroprevalences of IgG antibodies were 30·2% (461 of 1528) for HPV-16 and 16·0% (244 of 1528) for HPV-18 in Chinses women aged 18-25 years old (internal data), which were comparable to those obtained with ELISA conducted by GSK in Jiangsu, China (30·5% for HPV-16 and 16·0% for HPV-18) in the same age group.[23] Zhao H et al[21] also showed that when detecting unvaccinated serum samples, the agreements between our ELISA and the ELISA test from GSK were 0·87 and 0·83 for HPV-16 and HPV-18, respectively, which suggest a high level of agreement between the two ELISA tests. These data suggest that the original cut-off setting of our ELISA assay is acceptable and comparable with other widely used ELISAs, which also implies that the cut-off of IgG should be carefully reset for protection effect analysis. It should be noted that although the available data implicated that the characteristics of the ELISA used for this work doses not differ obviously from the other widely used ELISA test (by GSK), the possibility of lack of specificity and/or other limitations of the ELISA used in this study could not be absolutely excluded.

The extent of preventive immunity raised by natural HPV infection is one of the key parameters of vaccine cost-effectiveness analysis. Functional neutralizing antibody measured by PBNA is a more specific indicator for these protection investigations. However, PBNA is highly labour intensive and therefore seldom used in large epidemiological studies. ELISAs, which are high-throughput and easier to set up, are more practical and thus widely used in epidemiological studies; there is also high heterogenicity among different ELISAs because the method is influenced by the characteristics of coating HPV L1 antigen, which may be a reason for the fluctuation of the protection extent in different HPV natural immunity studies. A meta-analysis showed an approximately 35% and 30% decreased risk of subsequent infection with HPV-16 and HPV-18 among women who were seropositive for corresponding type IgG antibodies, respectively.[24] Based on the PBNA method, we showed that women with seropositive HPV-16/18 neutralizing antibodies had a significant 84% reduced risk of subsequent 6-month persistent infection, indicating that natural antibodies provide considerable protection against future infection. The findings provide a more objective parameter for the evaluation of vaccine cost-effectiveness.

One of the limitations in our study and other similar studies is the potential imbalance between seropositive and seronegative women in the risk of exposure to HPV due to differences in sexual behaviour and many other confounding factors. All participants in our study had 1-4 sexual partners at entry. We did not collect information about covariates, such as sexual behaviours and condom use, which are known to be associated with the risk of acquiring HPV. Nonetheless, some studies have shown that these covariates do not play an important role, with very similar results obtained with univariate and multivariable adjusted analyses.[16-18] A previous study suggested that women seropositive had a higher mean number of sexual partners and a higher incidence of a history of chlamydia infection.[15] Our data also showed that women seropositive for neutralizing antibodies were more likely to be diagnosed with cytological abnormalities. Thus, women seropositive for HPV-16/18 antibodies at baseline might be individuals with a higher behaviour risk of HPV infection, and the true preventive effects of antibodies might be underestimated in the above analyses. To determine whether this is true, we analysed the incidence of HPV-52 infection in women who were negative for HPV-52 DNA at baseline and with different HPV-16/18 serostatuses. HPV-52 is the most prevalent HPV type in healthy Chinese women,[25] with no or very limited cross-protection effect with HPV-16/18.[26] A 1·44-fold (95% CI: 1·08-1·91) higher risk of HPV-52 incident infection was observed in the HPV-16/18 Neu (+) subgroup than in the Neu (-) subgroup (Table S7 in the Supplementary Tables). Although not significant, the risks of persistent HPV-52 infection in the HPV-16/18 Neu (+) group were also higher than those in the HPV-16/18 Neu (-) group. By comparison, the risks of HPV-52 incident infection and persistent infection in the HPV-16/18 IgG (+) group were approximately the same as those in the IgG (-) group. Hence, the protective effect of neutralizing antibodies observed in our study might not be overestimated, which consolidates the conclusion that natural antibodies provide considerable protection against future infection.

Another limitation of our analysis is our inability to evaluate the effect of HPV neutralizing antibody titres; thus, we were not able to determine an accurate antibody threshold value for a defined reduction rate in infection. As the mean neutralizing antibody levels acquired from natural infection are very low (with 8·6 IU/ml for HPV-16 and 5·0 IU/ml for HPV-18 in women aged 18-26 years),[27] our data indicated that the protective antibody level of HPV might be even lower. Although a correlate of vaccine protection cannot be inferred from natural history studies, it would be plausible to allocate more resources to explore the one-shot vaccination programme for a quick reach of the global goal of eliminating cervical cancer.

In addition, it was not possible to determine whether an infection was a new infection or reactivation of a previous infection. Evidence exists that women can experience reactivation or redetection of a type-specific infection after a period of non-detection.[28] Based on this assumption, some infections considered new may indeed be persistent, which would bias the assessment of the relationship between natural antibodies and the risk of new infection. Furthermore, the protective role of naturally acquired HPV-16/18 antibodies in related cytology or disease was not clearly established due to limited power (small number of events in the follow-up) in the study.

In conclusion, our analysis showed that HPV natural immunity is considerably protective against future infection. Neutralizing antibodies are a highly specific indicator for HPV protective natural immunity, and binding IgG can serve as a surrogate indicator after setting a proper cut-off value. More studies are needed to explore the long-term protectiveness persistence and dynamics of natural immunities.

Declaration of Competing Interest

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Contributors

T Wu, Y Qiao, F Zhao, N Xia, J Zhang, X Yao and W Chen contributed to the study design. X Yao, W Chen, C Zhao, L Wei, Y Hu, M Li, Z Lin, B Lin, X Liu, Y Hong, Q Li, Q Pan, X Zhang, M Li, Y Zhao, L Zhang, H Xu, F Hu, J Zhao, Y Huang, W Sheng, Y Zheng, S Hu, S Huang and H Pan contributed to sample collection and experiment or data interpretation. X Yao, T Wu, Y Su, and J Zhang contributed to the data analysis and writing of the report. All authors reviewed or revised the manuscript and approved the final draft for submission.

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Data sharing statement

The data of the study are available for reasonable request for academic purpose. To gain access, please contact the corresponding author (Ting Wu, wuting@xmu.edu.cn) for further details.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.lanwpc.2021.100196.

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