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Antibodies reactive to *Plasmodium falciparum* serine repeat antigen in children with Burkitt lymphoma from Ghana

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Abstract

The role of protective immunity to *Plasmodium falciparum (Pf)* malaria in Burkitt lymphoma (BL) is unknown. We investigated the association between BL and antibodies reactive to SE36 antigen, a recombinant protein based on *Pf*-SERA5 gene, targeted by protective malaria immune responses. Cases were children (0–14 years) enrolled at the Korle-Bu Teaching Hospital, Accra, Ghana, during 1965–1994 with BL confirmed by histology or cytology (92% of cases). Controls were healthy appearing children enrolled contemporaneous to the cases from the nearest neighbor house to the case house age- and sex-frequency matched to the cases. Anti-SE36 IgG antibodies were measured using enzyme-linked absorbent immunoassays (ELISA). SE36 titers were estimated by extrapolating ELISA optical density (OD) readings to a standard fitting curve. Anti-SE36 titers were log-transformed for analysis. Odds ratios (OR) and 2-sided 95% confidence intervals (95% CI) were estimated using unconditional logistic regression. The mean log endpoint dilution titers were 0.63 logs lower in cases than in controls (8.26 [SD 1.68] versus 8.89 [SD 1.75], Student's t-test, P=0.019). Lower titers were observed in cases than controls aged 0–4 years (P=0.05) and in those aged 5–14 years (P=0.06). Low and medium tertiles of anti-SE36 IgG antibodies were associated with increased OR for BL ([OR 1.67, 95% CI 1.21–2.31] and [OR 1.33, 95% CI 0.96– 1.86], respectively, P_{trend} =0.002) in analyses adjusting for age, sex, calendar period, and test plate. Our findings suggest that compared to similarly aged children enrolled from the same community, children with BL in Ghana have lower antibodies to SE36 antigen.

Keywords

Plasmodium falciparum malaria; Africa; Epstein-Barr virus; immunity; epidemiology

Background

Burkitt lymphoma (BL) is an aggressive B cell lymphoma first described by Denis Burkitt in African children in 1958[1]. Epidemiological studies conducted shortly after linked BL to

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Plasmodium falciparum (*Pf*) malaria [2–4]. Specifically, a high incidence of BL was noted in regions where malaria transmission is high and a low incidence was noted in regions where malaria transmission is low[3, 5]. In addition, a reduction in BL incidence was reported in one community in Tanzania where malaria suppression intervention was applied[6], providing further epidemiological support for a role of malaria in BL. Recently, case control studies measuring anti-malaria whole schizont antibodies, which measure the cumulative impact of immune activation from malaria, have reported the odds of BL increase with increasing anti-schizont antibody titers[7, 8]. Biologically, malaria stimulates polyclonal proliferation of B cells, increases expression of Epstein-Barr virus (EBV) proteins, also linked to BL, and impairs EBV-specific T-cell immune responses [9], which may singly or jointly influence the risk for BL.

Environmental exposure to and subsequent infection with malaria is thought to be the most important determinant of individual risk for BL[5]. Whether intrinsic differences in level of protective immunity to mild clinical malaria or asymptomatic malaria parasitemic attacks might influence cumulative immunological burden from malaria among individuals from the same community has not been examined before. Lack of well established serological correlates of malaria immunity [10] have hitherto precluded this possibility from being examined. One study conducted in Ghana using age-, sex-, and residence-matched controls in 1979[11] found lower anti-malaria-specific IgG, IgA, and IgM antibodies. These findings were not replicated in a study conducted in Uganda during the same period[12]. Both studies were small, evaluated different populations and used assays whose malaria protective and growth inhibitory properties are uncertain. Recent efforts to develop malaria vaccines has led to characterization of several antigen targets for protective malaria immunity[13]. One of these is SE36; a recombinant protein based on the N-terminal domain of *P. falciparum* serine repeat antigen 5 (*Pf*-SERA5) genes. *Pf*-SERA5 exhibits no antigenic variation[14] and limited polymorphism[15] compared to other *Plasmodium* antigens, and aside from *Plasmodium Theileria* (the causative agent for East Coast fever in cattle) is the only genus to possess a SERA homolog gene[16]. Biologically, SERA expression appears to play an essential role in the release of invasive malaria parasites from host erythrocytes[17, 18]. SE36 is currently being evaluated as a blood stage vaccine candidate in clinical trials in Japan and Uganda [19]. Measuring SE36 would be a refinement of approaches relying on previously available whole schizont antigen, which has been used in previous case-controls studies that investigated the relationship between BL and malaria[7, 8]. Thus, we selected SE36 for our initial study to gain some insights on the immune-epidemiology of BL, specifically focusing on antibodies reactive to SERA5 in the Ghana BL case-control study conducted during 1965 to 1994[20]. A better understanding of malaria immunology in BL can provide information on the etiology of BL and help target BL treatment and/or prevention.

Study population

We used residual samples from the Ghana Burkitt lymphoma study conducted at Korle Bu Teaching Hospital in Accra, Ghana, during 1965 to 1994 (29 years)[20] to obtain preliminary data for our hypothesis. Briefly, the cases were children (0 through 14 years) enrolled from BL and malaria-endemic rural areas in the southern half of Ghana. Cases were confirmed by histology or cytology (92% of cases). Controls were apparently healthy children from the same community where the case arose. To find the controls, study staff visited the home of the case and starting from there, followed predetermined directions to reach the first home that was nearest to the home of the case and had children eligible to serve as controls. Eligible children were enrolled with frequency matching to the case on age and sex. Controls were enrolled contemporaneous to the case, except during 1980–1984 when it was interrupted leading to lower control numbers during that period. Some controls were members of the extended family of the case, but this group was thought to be small [21].

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Demographic (age, sex) information was collected from both cases and controls and venous blood was drawn; in the cases this was done before starting BL-specific treatment. Blood was processed within a few hours after collection and separated into sera, which was stored at −70°C until testing. The current study included sera from 657 (84%, of 778) cases and 498 (83% of 599) controls from the original study. Subjects were excluded either because sera were exhausted or cases have paired serum-tumor samples so their sera were preserved for future proteomic biomarker discovery studies. Parents or guardians of the children gave verbal informed consent for the children to participate and for blood samples to be taken and stored for use in future studies. The current study was done using anonymized data and samples that cannot be linked to original personal identifiers. Ethical approval for the current study was obtained from the Office of Human Subject Research at the National Institutes of Health.

Serological methods

Anti-SE36 IgG antibody were measured at the Research Institute for Microbial Diseases, Osaka University, Japan, using an enzyme-linked immunosorbent assay (ELISA) as previously described[22], with minor modifications. Sera (x100 dilution) were assayed twice for anti-SE36 IgG antibodies using flat-bottomed 96-well Nunc-Immuno plates (Nunc, Roskilde, Denmark) coated overnight at 4°C with 100 μL of antigen (recombinant SE36 protein) at a concentration of 1 μg/mL in carbonate coating buffer, pH 9.6. The plates were washed 3 times in PBS/0.05% Tween-20 (PBS/T)and blocked overnight at 4°C with 5% skimmedmilk powder in PBS/T. Prior to addition of sera, plates were again washed 3 times with PBS/T. Test sera were added(100 μL per well) at dilutions of 1:100 in 5% skimmedmilk powder inPBS/T and the plates were incubated overnight at 4°C. After washing thrice in PBS/T, horseradish peroxidase conjugated anti-human IgG (Horseradish peroxidase-conjugated rabbit anti-human IgG antibody A8792; Sigma-Aldrich Corp., St. Louis, MO) diluted 1:4000 in 5% skimmedmilk powder in PBS/T was added and the mixture incubated at room temperature for 4 hours. The plates were washed3 times, and color development reaction was done with TMB Microwell Peroxidase Substrate System (KPL, Inc., Gaithersburg, MD) for 1 minute. The reaction was stopped with 50 μL of2 M sulfuric acid and optical density (OD) read at 450 nm. Healthy malaria-naive Japanese serum was used as negative reference. Cutoff for positivity was set from mean OD values in negative controls + 3SD. For quantitation of antibody titers in the test sample, each plate contained a Ugandan high titer pool (made from a pool of 10 individuals from malaria-endemic region in Uganda) that was used as a standard and serially diluted from 1/100, 1/300, 1/900 to 1/218, 7000 (eight points from 100 to 218,700 dilution) to fit as a standard curve (Figure 1). OD values were fit on standard fitting curves to calculate SE36 antibody titers. We included 5% samples as blinded duplicates to measure variability in estimating endpoint dilution titers using this assay system.

Statistical methods

Endpoint dilution titers of anti-SE36 IgG antibodies were log-transformed (base 2) to obtain a normal distribution to facilitate analysis on a continuous scale. Log-transformed titers were grouped into a three-level ordinal scale (low, medium, and high titer) using cutoffs derived from the values in the control group, which represents, to some extent, the values that would be seen in the 'normal' population. The means of log-transformed anti-SE36 IgG antibodies for grouped variables (cases versus controls over all and by age [0–4 years and 5–14 years], early [0–2] versus late stage [3–4] disease among cases) were compared using the Student's t-test. We used two age groups that reflect extreme differences in malaria-related mortality, which is highest in children aged 0–4 years and substantially lower in children aged 5–14 years. Crude and adjusted odds ratios (OR) and 2-sided 95% confidence interval (CI) for association of BL with anti-SE36 antibodies were estimated using unconditional logistic

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regression because controls were frequency matched to the cases. ORs were adjusted for the matching variables age, sex, enrollment period, and for test plate, which included to the final model to adjust for imprecision of assay measurement across plates. Chi-squared *P* values for trend were used to assess for a trend across tertiles of anti-SE36 IgG antibody titers and Chi-squared *P* values for heterogeneity were calculated to assess whether at least one association for a multi-level category variable was statistically significant from the others. Because we used tertiles agnostically without presuming a biologically relevant cutoff point, we performed sensitivity analyses where individuals with medium anti-SE36 IgG antibody titers were either included as negatives or excluded from analyses. A two-sided *P*-value <0.05 was considered statistically significant.

Results

Males were more frequent than females among the cases and most cases were aged 5–9 years or 10–14 years (Table 2); cases and controls were enrolled during all calendar periods, but the last 10 years (1984–1994) of the study accounted for the majority (about 50%). The mean log endpoint dilution titer was 8.36 logs (range 1.71–15.2) for cases and controls combined. We noted some within-subject variation in anti-SE36 ELISA measurements, based on a coefficient of variation of 10% and 16%, based on 5% duplicate quality-control samples. The mean log endpoint dilution titer was 0.63 logs lower in cases than in controls (8.26 [SD 1.68] versus 8.89 [SD 1.75], p=0.019) overall (Figure 2A). It was 0.62 logs lower in cases than controls aged 0–4 years (8.25 [SD 1.71] versus 8.87 [SD 1.64], *P*=0.05) and 0.2 logs lower in those aged 5–14 years (8.26 [1.67] versus 8.46 [1.75], *P*=0.06). The mean log endpoint dilution titers in cases with early stage disease was similar to that of cases with late stage disease (8.29 [SD 1.54] versus 8.17 [1.63], *P*=0.47) (not shown). In analyses by calendar-year period, mean log titers were on average 0.2 logs lower in BL cases than in controls for all calendar periods (Figure 2B–D), but the strata-specific differences were not statistically significant.

The cutoff points for anti-SE36 IgG antibody titer tertiles derived from the control group were 7.7 and 9.3 logs. Compared to controls, cases were more likely to have low or medium than high anti-SE36 IgG antibody titers (OR 1.61, 95% CI 1.21–2.15], and [OR 1.27, 95% CI: 0.94–1.70], respectively, P_{trend}=0.001) (Table 1). The associations did not change substantially when we adjusted for sex, age, and calendar year of enrollment (results not shown) and it only changed slightly when test plate was included in the model (OR 1.67, 95% CI 1.21–2.31] and [OR 1.33, 95% CI: 0.96–1.86], respectively, P_{trend}=0.002). The association remained significant in sensitivity analyses where medium tertiles of anti-SE36 IgG antibody titers were coded as low (OR 1.42, 95% CI: 1.11–1.81) and when they were coded as indeterminate and excluded in the analysis (OR 1.61, 95% CI: 1.21–2.15).

Discussion

We hypothesized that serological response to SE36, an antigen that has been shown to elicit protective immunity to malaria[19], may vary between BL cases and controls enrolled from the same community in Ghana. We found modest but statistically significant lower mean log anti-SE36 titers in cases than in controls enrolled from the same community as the cases. We confirmed this result using categories agnostically grouped according to tertiles calculated from results obtained in the control group. Our results provide the first indication that humoral immune responses to an antigen targeted by protective malaria immunity, in this case SE36, may be lower in children with BL than controls from Ghana. Previous studies relied on the whole schizont anti-malaria antibodies, which measure the cumulative impact of malaria-related immune activation on the risk for BL[7, 8], but not protection from severe malaria. Antibodies reactive to SE36 antigen have been shown in previous

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immunoepidemiological studies to correlate with lower risk for malaria fever or parasitemia and protective immunity in Aotus and squirrel monkeys against parasite challenge [13, 14, 19]. Taken together, our study and previous case control studies[7, 8] support the role of malaria in BL, but they suggest that the association with BL may differ according to the association between the specific anti- malaria-antigen antibody and severe malaria. Our findings need to be confirmed in hypothesis-driven studies using a larger panel of malaria antigens targeted by humoral and other immune responses to malaria. In addition, proof of concept studies need to be done to define levels of humoral immune response that may significantly impact on the frequency of mild or asymptomatic malaria attacks, malaria parasitemia and to elucidate the role of malaria immunoepidemiology in BL.

Our study has some weaknesses. First, the case-control design raises concern that the findings may be the result, not the cause, of BL. This concern was alleviated, somewhat, by our finding similar mean titers in cases with early disease versus cases with late disease. Second, we evaluated only one malarial antigen targeted by malaria immunity and we did not measure the whole schizont anti-malaria antibody, whose association with BL is well established. This approach was considered reasonable to obtain preliminary data to support future hypothesis-driven studies. We did not have data on use of anti-malarial treatment prior to admission. Recent anti-malaria treatment is not known to suppress antibodies to the whole-schizont anti-malaria antibodies. An increased risk for BL was associated with elevated anti-malarial antibody titers in Uganda[7], although children with BL also frequently reported a history of treatment for malaria, suggesting that prior malaria treatment may not be associated with depression of anti-malaria antibodies. Third, samples from cases were collected at the hospital, whereas samples from controls were collected in the community, which may have resulted in slight differences in pre-processing times or conditions of the sera in cases versus controls. Efforts were made to process all samples the same day, usually within 2–8 hours, thus, differences in pre-processing conditions would have been minimal. Finally, the samples were collected over 29 calendar years (1965–1994), which may distort results due to differences in sample handling and storage or in changes in malaria epidemiology over time. Decay of antibodies is a theoretical concern, but is probably of less concern in or study as immunoglobulins are stable under a broad range of pre-analytic and long-term storage conditions[23, 24]. Long-term changes in malaria epidemiology would not undermine our results because malaria control activities during the study period were largely ineffective and malaria-specific mortality increased in most African countries in Africa [25], including in Ghana. The implementation of effective malaria control measures, including bed nets, insecticide spraying, and combinatorial treatments has only been introduced recently in the 2000s[26], so they would not impact our results. Anti-SE36 IgG antibody titers were lower in cases than controls for all calendar year periods, but period-specific analyses were under powered to show significance differences. Readers should consider these caveats when interpreting our results. Our results are valuable for generating new hypotheses, which can be tested in hypothesis-driven studies including adequate sample size and more malaria markers. The strengths of our study include using controls from the same community as the case and enrolling controls contemporaneous to the cases, especially because of data suggesting that environmental malaria in endemic areas can vary by geography and time[27].

We are aware of only one other study of BL that evaluated antibody assays that measure protective immunity to malaria in patients with BL[28], including the merozoite surface protein-1, which is being evaluated as a vaccine candidate [28]. No significant differences were noted in titers of the anti-MSP1 antibodies or the other anti-malarial antibodies evaluated between frequency age- and residence matched BL cases and controls[28]. Significant differences, however, were noted in anti-Zta and VCA EBV antibodies between BL cases and controls. The inconsistency with our study may be explained by the usage of different malarial antigens as well as study sample size (32 BL cases and 25 controls) which

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may have lacked power to exclude a positive result of a magnitude observed in our study (657 cases and 498 controls), or to over matching in their study. Because anti-SE36 antibodies were not assessed in that study, their findings are not directly comparable to our study. Large and well designed malariaimmuno-epidemiological studies are needed to confirm or refute our findings.

Although SE36 is an abundantly expressed late-trophozoite to schizont stage protein, we do not know why SE36 titers, which presumably represent more malaria experience, were associated with decreased risk for BL. Anti-SE36 IgG antibodies have been associated with reduced risk of severe clinical malaria in children in Uganda [22] exposed to extraordinarily high environmental malaria (1500 or up to 5 infectious mosquito bites per night[29]), evidence for malaria parasite growth inhibitory activity *in vitro*[14], and protection of non-human primates vaccinated with SE36 antigen[13, 19]. Possibly, genetic, nutritional[30], or environmental factors predispose some individuals to high anti-SE36 IgG titers resulting in a lower propensity for mild or asymptomatic malaria parasitemia. Conversely, other individuals lacking those genetic or environmental exposures may be predisposed to low anti-SE36 antibody titers resulting in a greater propensity for mild or asymptomatic malaria parasitemia, higher cumulative exposure to malaria-induced immune-activation. These explanations would be consistent with both the malaria hypothesis and with immunoepidemiological studies showing a correlation between BL and high titers of whole schizont malaria antibodies[7, 8].

To conclude, we found that compared to controls from the same community in rural Ghana, children with BL were more likely to have lower mean or low and medium anti-SE36 IgG antibody titers. Our findings suggest that serological reactivity to SE36 may be lower in children with BL in Ghana and suggest that it might be worth investigating these patterns in other settings or using a larger panel of antigens.

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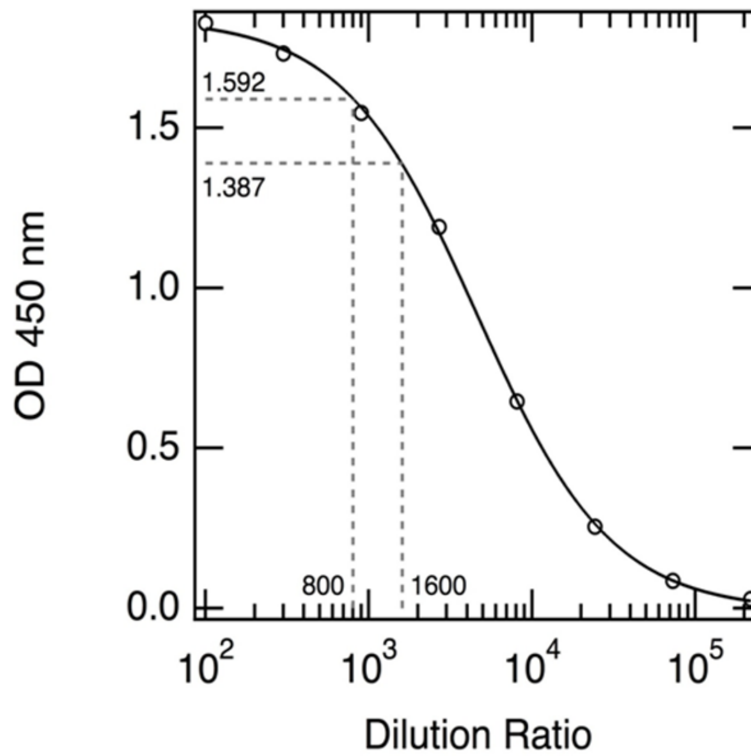

Figure 1.

Representative standard fitting curve for extrapolating optical density readings to estimated titers; Table shows standard endpoint titer dilution, and theoretical titers for plate 4. Serum dilution based on a pool of strongly reactive malaria-smear positive sample from 10 Ugandan individuals.

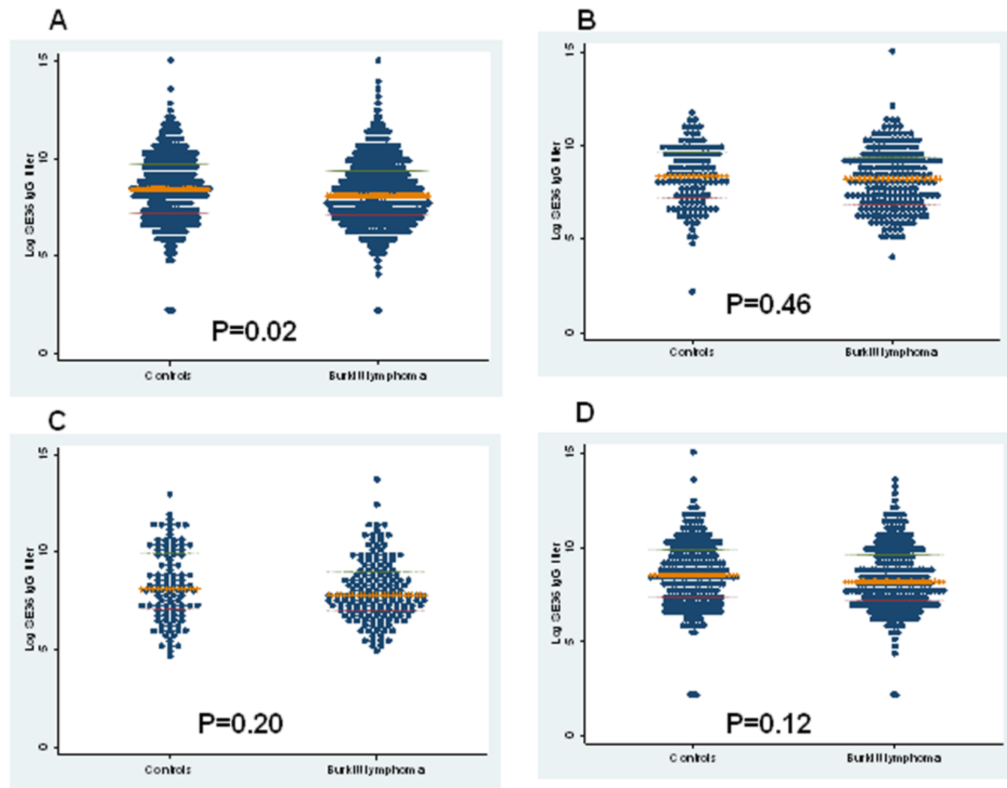

Figure 2.
Dot plot of log (base 2) anti-SE36 IgG antibody titers for all cases and controls (panel A) and for cases and controls during 1965–1974 (Panel B), 1975–1984 (Panel C) and 1985–1994 (Panel D). Each dot represents a single subject, the horizontal bar in the middle is the median and the outer bars represent the 25th and 75th inter-quartile ranges.

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Table 1

Standard fitting curve

	Standard dilution ratio	Optical density readings	Calculated Titer	Theoretical Titer
H1	100	1.339	111721.176	218700
H2	300	1.359	157952.877	72900
H3	900	1.08995	22121.054	24300
H4	2700	0.74355	7875.525	8100
H5	8100	0.39525	2992.76	2700
H6	24300	0.10145	693.254	900
H7	72900	0.04785	347.159	300
H8	218700	0.01445	130.911	100

Table 2

Characteristics of Burkitt lymphoma cases and controls 0–14 years from Ghana, 1965–1994

	Cases (N=657)		Controls (N=498)		Crude Odds Ratio (95% CI)	Adjusted Odds* Ratio (95% CI)
	n	%	n	%		
Sex						
Female	250	38.1	227	45.6	Reference	Reference
Male	407	61.9	271	54.4	1.36 (1.08, 1.73)	1.30 (1.02, 1.66)
Age Group, years						
0–4	96	14.6	41	8.2	1.46 (0.98, 2.18)	1.45 (0.97, 2.18)
5–9	391	59.5	244	49.0	Reference	Reference
10–14	170	25.9	213	42.8	0.50 (0.39, 0.64)	0.50 (0.39, 0.65)
Enrollment year						
1965–1974	200	30.5	119	23.9	1.54 (1.17, 2.04)	1.52 (1.14, 2.02)
1975–1984	152	23.1	99	19.9	1.41 (1.04, 1.90)	1.38 (1.01, 1.87)
1985–1994	305	46.4	280	56.2	Reference	Reference
Anti-SE36 IgG antibody titers						
High tertiles	168	25.6	165	33.1	Reference	Reference
Medium tertiles	217	33.1	168	33.7	1.27 (0.94–1.70)	1.33 (0.96–1.86)
Low tertiles	271	41.3	165	33.1	1.61 (1.21–2.15)	1.67 (1.21–2.31)

* Model includes all variables in the table and plate to plate variation.

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