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Epstein-Barr virus and risk of non-Hodgkin lymphoma in the cancer prevention study-II and a meta-analysis of serologic studies

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Epstein-Barr virus (EBV) causes rare, malignant lymphomas. The role of EBV in other non-Hodgkin lymphomas (NHLs) remains unclear, but mildly reduced immune function could lead to reactivation of EBV and subsequent NHL. We examined the association between prospectively-collected plasma EBV antibodies and NHL risk in the Cancer Prevention Study-II (CPS-II) Nutrition Cohort and conducted a meta-analysis of our and published results. The CPS-II study included 225 NHL cases and 2:1 matched controls. No associations were observed between EBV serostatus or antibody levels and risk of NHL overall. However, when including only the three most common types of NHL (diffuse large B-cell lymphoma, follicular lymphoma, chronic lymphocytic leukemia/small lymphocytic lymphoma), high compared to low early antigen (EA-D) diffuse and BZLF1-encoded replication activator antibodies were associated with approximately 60% higher risk of NHL. Odds ratios (ORs) for EBV nuclear antigen-1 and viral capsid antigen (VCA)-p18 were elevated but not statistically significant. In the meta-analysis, both EA (summary OR = 1.52, 95% confidence interval (CI): 1.16–2.00) and VCA (summary OR = 1.20, 95% CI: 1.00–1.44) were positively associated with NHL risk. These results suggest EBV may be associated with a wider spectrum of NHL subtypes, but further study is needed to confirm and fully understand these associations.

Epstein-Barr virus (EBV) is a known carcinogen and has been causally linked to some rare types of non-Hodgkin lymphoma (NHL) and NHL in immunosuppressed individuals.[1] In particular, the 2009 IARC Working Group on the carcinogenicity of biologic agents found sufficient evidence to conclude causality for EBV's role in Burkitt lymphoma, immunosuppression-related NHL, extranodal NK/T-cell lymphoma (nasal type), Hodgkin lymphoma and cancer of the nasopharynx. The role of EBV in more common types of NHL and in seemingly immunocompetent individuals is less clear, particularly since EBV prevalence in adult humans is >90%, and EBV establishes lifelong latent infection in memory B-cells. The mechanism by which HIV-1 positive, immunocompromised individuals are at higher risk of NHL is thought to be mostly due to immunosuppression leading to increased replication of EBV and other carcinogenic viruses.[2] It is possible that more mild forms of reduced immune function may lead to reactivation and replication of EBV. For example, psychological stress and aging can dysregulate immune function[3] and have been implicated in subclinical reactivation of EBV.[4,5] EBV reactivation itself may be a risk factor for NHL, alternatively the external factor responsible for reducing immune function may cause both EBV reactivation and an increase in risk of NHL. Either way, we would expect the higher antibody levels that accompany reactivation (rather than seropositivity) to be associated with subsequent risk of NHL. Results of serologic studies of EBV antibodies and NHL risk published to date are inconsistent.[6–11] This inconsistency might be due, in part, to the limited sample size in most studies, and differences in EBV antibodies assessed.

In this analysis, we examined the associations of antibody levels for several commonly assessed EBV antigens with the subsequent incidence of NHL and NHL subtypes over 8 years of follow-up, using plasma samples archived in the Cancer Prevention Study-II (CPS-II) Nutrition Cohort. Because of the limited power in studies on this topic, we also conducted

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What's new?

People with weakened immune systems can fall victim to non-Hodgkin's lymphoma caused by unchecked Epstein-Barr virus. But can the virus cause cancer in people without obvious immune deficiencies? The vast majority of us harbor EBV, and it could be that even a mild immune impairment allows reactivation of the virus and increased risk of cancer. In this study, the authors examined the association between EBV antibodies and NHL risk. When considering the three most common types of NHL, they found that certain EBV antibodies did associate with increased risk, suggesting the virus may cause more cancers than previously thought.

a meta-analysis of our and published results to better understand whether EBV antibodies are associated with NHL risk.

Material and Methods

CPS-II nutrition cohort

Study population. Men and women in this nested case–control study were among the 184,194 participants in the CPS-II Nutrition Cohort, a prospective study of cancer incidence established in the United States in 1992. Follow-up questionnaires were sent to cohort members in 1997 and every 2 years thereafter to ascertain newly diagnosed cancers and collect updated exposure information. For all follow-up cycles, response rates were at least 87% among participants who were mailed follow-up questionnaires. From 1998 to 2001, participants in the CPS-II Nutrition Cohort were invited to provide a blood sample at a medical facility in their community. At the time of blood collection, CPS-II participants were aged 47–95 years. Eligible participants were defined as CPS-II Nutrition Cohort participants who (*i*) were believed to be alive at the time blood collection began in their area (*ii*) had not reported a cancer diagnosis during the interval 1992–1997 and (*iii*) were residents of urban and suburban areas in 20 states included in the CPS-II Nutrition Cohort. Of the 129,231 potential participants, non-fasting blood samples were provided by 39,371 participants. At the time of blood draw all participants completed a brief questionnaire and provided informed consent. Blood samples were collected in two 15-ml tubes containing the anti-coagulant EDTA and in a 13-ml serum separator tube. The tubes were kept chilled while shipped overnight to a central repository in Rockville, Maryland where they were then separated into plasma, serum, red blood cells and buffy coat fractions, aliquoted, and finally placed in liquid nitrogen freezers for long-term storage. The recruitment, characteristics and follow-up of the CPS-II Nutrition Cohort are described in greater detail elsewhere.[12] All aspects of the CPS-II cohort were approved by the Emory University Institutional Review Board.

Case ascertainment. Among the 39,371 previously cancer-free participants who provided a blood sample between 1998 and 2001, 225 participants (129 male and 96 female) were subsequently diagnosed with NHL through June 2007. Incident NHL cases reported on biennial CPS-II Nutrition Cohort questionnaires were verified through medical records ($n = 169$) or linkage with state cancer registries ($n = 56$). NHL cases were defined using the *International Classification of Disease for Oncology, Second and Third Editions* (ICD-O-2 and −3) based on the 2008-revised WHO classification of tumors of hematopoietic and lymphoid tissues,[13] and were grouped into the following subtypes: diffuse large B-cell lymphoma (DLBCL; ICD-O: 9,680; $n = 67$), follicular lymphoma (ICD-O: 9,690, 9,691, 9,695, 9,698; $n = 46$) and chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL; ICD-O: 9,670, 9,823; $n = 66$). The remaining 46 cases had uncommon or unspecified histologies: marginal zone (ICD-O: 9,689, 9,699; $n = 14$), T-cell (ICD-O: 9,684, 9,700, 9,701, 9,702, 9,709; $n = 10$), mantle cell (ICD-O: 9,673; $n = 5$), lymphoplasmacytic lymphoma (ICD-O: 9,671, 9,761; $n = 10$), precursor lymphoblastic leukemia/lymphoma (ICD-O: 9,835; $n = 1$) and NHL not otherwise specified (ICD-O: 9,591; $n = 6$).

Control selection. For each eligible case, we randomly selected two matched controls from participants who provided a blood sample, were alive and had no personal history of cancer as of the diagnosis date of the case. Each control was also individually matched to its case on birth date (±6 months), blood draw date (±6months), sex (male/female) and race (white, black, other/unknown). The birth and blood draw date criteria had to be relaxed to within 1 year for one black female case and her controls as it was not possible to get two matched controls within 6 months.

Antibody measurements. Serological analyses to measure antibodies to the viral proteins were conducted at the German Cancer Research Center in Heidelberg, Germany. Frozen plasma samples were shipped on dry ice. The antigens selected for analysis are representative of the different infection phases (*i.e.*, primary infection, latency and reactivation), and they were chosen to increase the sensitivity of the EBV infection detection as well as potentially allow differentiation of infection stages.[14] Immunoglobulin G (IgG) antibodies to viral capsid antigen (VCA) are produced within a few days of primary EBV infection and peak after 3–4 weeks. Subsequently these antibodies decline slowly but remain present throughout the lifespan. Antibodies to EBV nuclear antigen (EBNA) are characteristically expressed in subacute stages of disease, and like VCA antibodies, persist indefinitely. BZLF1-

encoded replication activator (ZEBRA) is expressed during the lytic cycle in EBV-permissive cells and antibodies are produced during primary EBV infection. ZEBRA is a key mediator of the switch from latency to productive cycle in EBV and therefore a marker of viral reactivation. Antibodies to early antigen (EA) appear transiently for up to 3 months during the acute phase of infectious mononucleosis. Upon reactivation of EBV due to immunosuppression, antibodies to EA rise moderately and remain elevated in chronic infection.

Seroreactivity against full-length EA-diffuse (EA-D), VCAp18, ZEBRA (all EBV strain M-ABA) and a fragment of EBNA-1 (C-terminal part AA 325–641, EBV strain B-95-8),[14] were measured by fluorescent bead-based multiplex serology and quantified as median fluorescence intensity (MFI). This method has been described in detail elsewhere.[14–16] Briefly, full-length viral proteins or proteins fragments were expressed in bacteria in fusion with an N-terminal glutathione S-transferase (GST) domain. Glutathione cross-linked to casein was covalently bound to fluorescence labeled polystyrene beads (SeroMap; Luminex), and GST-fusion proteins were affinity-purified on the beads directly. Bead sets of different colors each carried a different antigen and were mixed with human plasma at 1:1,000 dilutions. Antibody bound to the beads *via* the viral antigens was stained by biotinylated anti-human-Ig and streptavidin-R-phycoerythrin. Beads were examined in a Luminex 100 analyzer that identifies the different bead types by their internal color and quantifies the antibody bound to the viral antigen on the different bead types *via* the median R-phycoerythrin fluorescence intensity of at least 100 beads of each bead type. To assess specificity of the associations between the EBV antibodies and NHL risk, antibodies to BK polyomavirus, which also has a high population seroprevalence (~90%), was also measured.

A total of ten participants (six males and four females) were selected for use as quality controls (QCs). Fifty-six blinded QC samples (six replicates from each of eight samples and four replicates from each of two samples) were assayed at the same time as the case and control samples. Because the MFI values were not normally distributed, we log transformed the data to do the QC analyses. The QC results were as follows: coefficients of variation for these replicate samples were 3% for ZEBRA, 4% for EBNA-1, 8% for EA-D and 2% for VCAp18; and the intra-class correlation coefficients were 99% for ZEBRA, 94% for EBNA-1, 96% for EA-D and 95% for VCAp18.

EBV seropositivity was defined as antibody-positivity (ZEBRA, EA-D, VCAp18: MFI $>$ 100; EBNA-1: MFI $>$ 250) against two or more measured antigens. Antibody levels among the EBV seropositive participants were divided into three groups based on the distribution of each antigen among seropositive controls: MFI $<$ 25th percentile, MFI 25th–75th percentile and MFI $\geq$ 75th percentile.

Statistical methods. Odds ratios (ORs) and 95% confidence intervals (CIs) were estimated using conditional logistic regression modeling. Models for NHL overall were conditioned on matched pair, thereby adjusting for the matching factors—sex, draw date, birth year and race. Further adjustment for other potential confounders including body mass index, alcohol intake, cigarette smoking status and geographic region of residence, did not appreciably alter associations between any of the EBV antibody levels and NHL risk. Restricted cubic splines were used to examine possible non-linear associations.[17] Tests for non-linearity used the likelihood ratio test comparing the model with only the linear term to the model with the linear and the cubic spline terms. Due to sample size constraints, analyses of the NHL subtypes were done with unconditional polytomous logistic regression. A sensitivity analysis excluding the first 3 years of follow-up was conducted to evaluate the potential impact of reverse causality, that is, the possible effects of preclinical lymphoma on antibody levels.

Meta-analysis

Study selection. Seven[6–11,18] epidemiologic studies investigating the association between serum or plasma EBV antibodies and NHL, published in English language in peer-reviewed journals through October 2013 were identified in PubMed searches and references of identified papers. In addition, we included unpublished ORs for EBV serology provided by de Sanjose *et al.*[19] using the case–control population referenced in their 2007 manuscript on antibody responses to EBV-related proteins. Results from the CPS-II Nutrition Cohort study described above also are included. Among the included studies, three were population-based case–control studies[7,8,19] and six were case–control studies[6,9–11,18] nested within prospective cohorts (including CPS-II).

Data extraction. The following information was abstracted from each study: the first author's last name, publication year, country where the study was performed, study period, sex and age of study participants, number of cases and controls, antigens measured, method for assessing antigen level, study-specific OR and the corresponding 95% CI for seropositivity as well as "high" antibody level (where applicable) and variables adjusted for in the analysis (Table 3). When several risk estimates were presented for a given study, those adjusted for the largest number of potential cofounders were included in the meta-analysis.

Statistical analysis. Summary OR estimates were calculated with a random-effects model which considers both within- and between-study variability. Statistical heterogeneity among studies was evaluated by using both Cochran's *Q*-test for heterogeneity[20]and the I^2 (the proportion of variation in the OR attributable to heterogeneity).[21] Potential publication bias was assessed using the Begg[22] test using STATA SE (version 11.0). All other statistical analyses were performed with SAS 9.3. $p < 0.05$ was considered statistically significant.

Results

CPS-II nutrition cohort

We excluded three controls from the final analysis because of high anti-GST antibody reactions leading to an inappropriate

Table 1. Characteristics of NHL cases and controls in the CPS-II Nutrition Cohort

Characteristics	Cases ($n = 225$) *N* (%)	Controls ($n = 449$) *N* (%)	*p*-Value
Male gender	129 (57.3)	257 (57.2)	0.98
Smoking status			
Nonsmoker	106 (47.1)	231 (51.4)	0.47
Current smoker	5 (2.2)	16 (3.6)	
Former smoker	107 (47.6)	191 (42.5)	
Unknown	7 (3.1)	11 (2.4)	
Current alcohol use			
Non-drinker	70 (31.1)	170 (37.9)	0.50
< 1 drinks/day	91 (40.4)	168 (37.4)	
1–2 drinks/day	39 (17.3)	64 (14.3)	
>2 drinks/day	22 (9.8)	40 (8.9)	
Unknown	3 (1.3)	7 (1.6)	
Geographic region (US)			
MidWest	66 (29.3)	156 (34.7)	0.32
Northeast	57 (25.3)	122 (27.2)	
South	42 (18.7)	70 (15.6)	
West	60 (26.7)	101 (22.5)	
Body mass index (BMI, kg/m^2)			
<25.0 (under/normal weight)	94 (41.8)	200 (44.5)	0.48
25.0–<30.0 (overweight)	88 (39.1)	178 (39.6)	
≥30.0 (obese)	42 (18.7)	66 (14.7)	
Unknown	1 (0.4)	5 (1.1)	
Education			
Less than high school	7 (3.1)	18 (4)	0.72
High school/missing education	47 (20.9)	81 (18)	
Some college/vocational school	52 (23.1)	115 (25.6)	
College/graduate school	119 (52.9)	235 (52.3)	
Mean age (years) at blood collection (SD)	70 (5.55)	70.1 (5.58)	0.80
Mean years between blood collection and NHL (SD)	3.7 (2.08)	–	

signal-to-noise ratio. After this exclusion, one case was left with no controls and was also excluded. The final study population was 674 participants (225 cases with two matched controls and one case with only one matched control). Cases were diagnosed with NHL between 0.1 and 8 years after blood collection (median 3.7 years). Cases were more likely to be obese, former smokers, drink alcohol and live in the United States South compared to controls (Table 1).

Though most participants ($n = 628$) were classified as seropositive for EBV (94% of cases and 93% of controls), seropositivity varied for the four different EBV antigens: ZEBRA: 88%, EBNA-1: 92%, EA-D: 80%, VCAp18: 93%. No association was observed between EBV serostatus and risk of NHL overall (OR = 1.28, 95% CI: 0.67–2.47) (Table 2). Likewise, no statistically significant association was observed for high (≥75th percentile among controls) compared to low (< 25th percentile among controls) levels of any of the individual antibodies measured and risk of NHL overall. Evaluation of restricted cubic splines did not suggest any nonlinearity in the associations of EBV antibody levels and NHL risk (data not shown). The time since blood collection sensitivity analysis suggested a twofold higher risk of NHL for seropositivity three or more years after blood collection (OR = 2.26, 95% CI: 0.84–6.05) and an inverse association within 3 years of blood collection (OR = 0.67, 95% CI: 0.26–1.72), but these results were based on small numbers of seronegative cases and were not statistically significant. In addition, time since blood collection was not associated with any of the individual antibody levels (data not shown). There was no association between BKV seropositivity and NHL risk (+ *vs.* −: OR = 0.83, 95% CI: 0.53–1.29), nor were there any associations between antibody level and risk.

Table 2. Association of EBV serostatus and antibody level with risk of all NHL and common NHL subtypes in the CPS-II Nutrition Cohort

		All NHL[1]		DLBCL[2]		FL[2]		CLL/SLL[2]		Other NHL[2]	
	Controls	Cases	OR (95% CI)	Cases	OR (95% CI)	Cases	OR (95% CI)	Cases	OR (95% CI)	Cases	OR (95% CI)
EBV serostatus											
Negative	33	13	1.00 (ref)	2	1.00 (ref)	4	1.00 (ref)	5	1.00 (ref)	2	1.00 (ref)
Positive	416	212	1.28 (0.67–2.47)	65	2.63 (0.61–11.28)	42	0.83 (0.28–2.50)	61	0.91 (0.34–2.45)	44	1.69 (0.39–7.34)
EA-D											
<25th	184	77	1.00 (ref)	26	1.00 (ref)	12	1.00 (ref)	18	1.00 (ref)	21	1.00 (ref)
25–75th	177	104	1.41 (0.98–2.02)	29	1.19 (0.67–2.11)	24	2.12 (1.02–4.41)	30	1.57 (0.84–2.95)	21	0.99 (0.52–1.90)
>75th	88	44	1.18 (0.76–1.85)	12	0.95 (0.45–1.99)	10	2.05 (0.83–5.06)	18	2.01 (0.98–4.13)	4	0.37 (0.12–1.11)
VCAp18											
<25th	131	61	1.00 (ref)	12	1.00 (ref)	15	1.00 (ref)	15	1.00 (ref)	19	1.00 (ref)
25–75th	212	115	1.17 (0.79–1.72)	38	2.02 (1.01–4.03)	20	0.87 (0.42–1.76)	35	1.48 (0.77–2.83)	22	0.68 (0.35–1.33)
>75th	106	49	0.99 (0.62–1.57)	17	1.79 (0.82–3.94)	11	0.92 (0.40–2.09)	16	1.31 (0.62–2.79)	5	0.31 (0.11–0.85)
EBNA-1											
<25th	142	72	1.00 (ref)	14	1.00 (ref)	12	1.00 (ref)	26	1.00 (ref)	20	1.00 (ref)
25–75th	205	91	0.87 (0.59–1.28)	25	1.25 (0.63–2.51)	21	1.19 (0.56–2.52)	26	0.64 (0.35–1.17)	19	0.66 (0.34–1.30)
>75th	102	62	1.23 (0.78–1.93)	28	3.02 (1.49–6.10)	13	1.34 (0.58–3.11)	14	0.64 (0.31–1.30)	7	0.46 (0.19–1.15)
ZEBRA											
<25th	153	76	1.00 (ref)	22	1.00 (ref)	15	1.00 (ref)	17	1.00 (ref)	22	1.00 (ref)
25–75th	197	90	0.93 (0.64–1.34)	28	1.01 (0.55–1.85)	19	1.01 (0.49–2.09)	27	1.16 (0.60–2.24)	16	0.53 (0.27–1.06)
>75th	99	59	1.21 (0.79–1.87)	17	1.21 (0.60–2.43)	12	1.40 (0.61–3.23)	22	1.90 (0.94–3.85)	8	0.48 (0.20–1.16)

[1]Conditional logistic regression (models stratified by matched pairs).
[2]Unconditional polytomous logistic regression.
Abbreviations: EBV: Epstein-Barr virus; EA-D: early antigen diffuse; VCAp18: viral capsid antigen p18; ZEBRA: BZLF1-encoded replication activator; EBNA-1: Epstein-Barr virus nuclear antigen 1; OR: odds ratio; CI: confidence interval; NHL: non-Hodgkin lymphoma; DLBCL: diffuse large B-cell lymphoma; FL: follicular lymphoma; CLL/SLL: chronic lymphocytic leukemia/small lymphocytic lymphoma.

Table 3. Serologic studies of EBV antibodies and NHL

Author	Type	Cases	Controls	Lab method	Definition of elevated EBV	Cutpoints	Elevated EBV OR
Mueller *et al.*[10] US, Norway	Nested case–control	104	259	Immunofluorescence	Top 15th percentile in the controls	VCA IgG (1:320) VCA IgA (1:40) VCA IgM (1:5)[1] EA-D (1:10) EA-R (1:80) EBNA (high) (1:160) EBNA (low) (1:5)	2.4 (1.2–5.0) 1.1 (0.5–2.3) 2.8 (1.3–6.0) 1.6 (0.7–3.4) 1.8 (0.9–3.6) 0.6 (0.3–1.6) 0.4 (0.2–1.0)
Lehtinen *et al.*[9] Finland	Nested case–control	11	22	ELISA	Higher absorbance value than that of the positive reference sera (>100 EIU)	VCA > 100EIU EA > 100EIU EBNA>100EIU	0.6 (0.1–7.4) 5.3 (0.6–50.0) 2.3 (0.2–24.4)
[2]Rollison *et al.*[11] US	Nested case–control	84	148	Immunofluorescence	EA antibodies: titers ≥20 VCA: titers ≥640	EA titer≥20 VCA titer≥640	2.42 (1.17–4.99) 1.44 (0.79–2.60)
Hardell *et al.*[7] Sweden	Case–control	67	78	Immunofluorescence	Median titer in the controls cutoff (≤ or >)	VCA IgG > 1,280 VCA IgM > 20 EA IgG > 80 P107 IgG > 1.5 P107 IgM > 0.17	1.6 (0.76–3.3) 0.42 (0.13–1.2) 1.9 (0.94–3.8) 0.68 (0.34–1.4) 0.81 (0.40–1.6)
de Sanjose *et al.*[19] Spain	Case–control	343	587	ELISA	Top 15th percentile of the seropositive controls	VCA EBNA	1.05 (0.73–1.51) 0.96 (0.63–1.46)
Hardell *et al.*[8] Sweden	Case–control	99	99	Immunofluorescence	Median titer in the controls cutoff (≤ or >)	VCA IgG > 2,560 EA IgG > 40	1.6 (0.8–3.0) 2.4 (1.3–4.3)
Bertrand *et al.*[6] US	Nested case–control	340	662	Immunofluorescence	Top 15th percentile of the seropositive controls	VCA (1: 10,240) EA (1:640) EBNA1 (1:320) EBNA2 (1:80) EBNA1/2 ratio >1	1.27 (0.84–1.93) 0.97 (0.66–1.42) 1.07 (0.76–1.52) 1.06 (0.74–1.52) 1.07 (0.79–1.44)
De Roos *et al.*[18] US	Nested case–control	491	491	Multiplex Serology	Top 25th percentile of the controls	VCA IgG ≥6.50 EA IgG ≥3.11 EBNA1 ≥3.64	0.9 (0.6–1.4) 1.4 (0.9–2.1) 1.1 (0.7–1.6)
Teras *et al.* US	Nested case–control	225	449	Multiplex Serology	Top 25th percentile of the seropositive controls	ZEBRA EBNA1 EA-D VCAp18	1.18 (0.76–1.83) 1.14 (0.74–1.75) 1.19 (0.75–1.88) 0.99 (0.63–1.57)

[1]VCA IgM presence alone was considered elevated.
[2]Ninety-six blood samples from cases and 167 blood samples for controls were used in this analysis.

In analyses to explore heterogeneity by NHL subtypes, the strongest association with serostatus was for DLBCL (OR = 2.63, 95% CI: 0.61–11.28) but this result, as well as those for the other NHL subtypes, lacked statistical significance (Table 2). ORs were elevated for the associations of the highest levels of three of the four EBV antibodies (EA-D, ZEBRA and EBNA-1) with follicular lymphoma, but none were statistically significant. ORs for the associations of the highest levels of EA-D and ZEBRA with CLL/SLL also were elevated but of borderline statistical significance. Similarly, ORs for mid-range (25th–75th percentile among controls) anti-EA-D and anti-ZEBRA suggested a dose–response relationship for both follicular and CLL/SLL, but the point estimate was only statistically significant for follicular lymphoma. High EBNA-1 and mid-range VCA were associated with DLBCL, otherwise these antigens were not statistically significantly associated with risk of any NHL subtypes. A strong inverse association was observed for all four antigens with "other" NHL. In contrast, BKV results were null regardless of which NHL subtypes were included in the analysis.

Meta-analysis

The meta-analysis included eight studies published between 1991 and 2013 and the CPS-II Nutrition Cohort study described above; together these studies included 4,559 study participants and 1,764 NHL cases (Table 3). In addition to the CPS-II Nutrition Cohort, three studies were conducted in the United States,[6,11,18] four in Europe,[7–9,19] and one in both

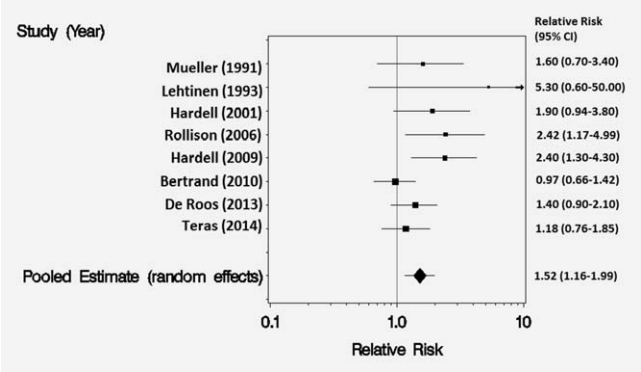

Figure 1. Relative risk estimates of NHL incidence for elevated EBV EA, for individual studies and all studies combined.

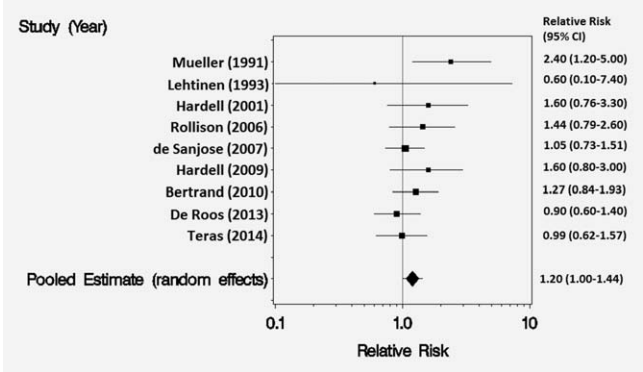

Figure 2. Relative risk estimates of NHL incidence for elevated EBV VCA, for individual studies and all studies combined.

the United States and Europe.[10] Five studies used immunofluorescence assays to measure IgG antibodies to EBV antigens,[6–8,10,11] two studies used ELISA,[9,19] and our study and one additional study[18] used multiplex serology as described above. All three of these methods have been shown to produce comparable antibody measurement results.[16,23] Most studies reported elevated ORs for both EA and VCA with risk of NHL. Fewer studies measured EBNA, and the specific viral protein measured (*e.g.*, EBNA-1 and EBNA-2), as well as the methods used to measure EBNA seroreactivity, varied across studies. As a result, the measures were too heterogeneous to include in the meta-analysis. In general, cutpoints for measured antibody levels varied among studies, but most used a dichotomous cutpoint at either the top 15th percentile or the median titer value. Lehtinen *et al.* reported an OR that included NHL, CLL and Hodgkin lymphoma, as well as NHL without CLL. As EBV is considered a causal agent for Hodgkin lymphoma,[1] we decided not to include the all-inclusive OR despite the more stable sample size. All studies controlled for age at blood draw and sex.

Figure 1 shows the study-specific and summary OR for EA-D and NHL. For the individual studies, most of the point estimates were greater than the null value but only two were statistically significant.[8,11] Neither the *Q*-test ($p = 0.12$) nor the I-squared test (39.27%) indicated high heterogeneity among the study results. Results of the meta-analysis showed a statistically significant positive association between EA-D levels and NHL risk (summary OR = 1.52, 95% CI: 1.16–2.00). For VCA, the individual study results were not as strong as for EA, but there was also no statistical evidence of heterogeneity for this antigen (*Q*-test: $p = 0.37$, I-squared test: 8.41%). As shown in Figure 2, there was a positive summary OR for VCA and NHL risk (summary OR = 1.20, 95% CI: 1.00–1.44). When only prospective studies (1,255 cases, 2,031 controls) were included, the summary OR for EA-D was slightly attenuated (OR: 1.35, 95% CI: 1.02–1.78) but was similar for VCA (OR: 1.20, 95% CI: 0.92–1.57). The Begg's test did not show evidence of publication bias for the EA ($p = 0.17$) or VCA ($p = 0.33$) results.

Discussion

In the CPS-II Nutrition Cohort, a prospective study of presumed immunocompetent men and women, there was no association between EBV serostatus or elevated EBV antibodies and NHL risk overall. The meta-analysis also showed statistically significant modest positive associations of EA-D as well as VCA antibodies with risk of NHL overall. Together these results support a possible broader role of EBV in the etiology of NHL than was evident when the literature was last reviewed by IARC in 2009.

The observed associations with EA (CPS-II and the meta-analysis) and ZEBRA (CPS-II) are consistent with the hypothesis that reactivation rather than initial EBV infection is responsible for lymphomagenesis.[1] During reactivation of EBV, antibodies to EA rise moderately and remain elevated in chronic infection. Likewise ZEBRA is an important mediator of the switch from the latent to the productive EBV cycle.[24] A positive association between EA antibodies and NHL risk is consistent with most[7–9,11] but not all[6,10] other studies that examined this association. To our knowledge, no other serology study reported results for ZEBRA and NHL risk. However, an immunoblot analysis examining EBV antibody patterns and NHL risk,[19] showed a positive association for abnormal EBV antibody patterns (*e.g.*, high EA and/or ZEBRA) with NHL risk (OR: 1.42, 95% CI: 1.15–1.74).

In analyses of specific NHL subtypes, elevated EBV antibody levels (EA and ZEBRA in particular) were most strongly associated with CLL/SLL and follicular lymphoma, though many of the point estimates were not statistically significant. To our knowledge, only three other studies reported serum/plasma EBV results separately for NHL subtypes.[6,11,18] Like CPS-II, Bertrand *et al.*[6] reported borderline positive associations between EBV antibodies and risk of CLL/SLL, but the specific antigens (EBNA-2, VCA and EBNA-1/EBNA-2 ratio) were different from the ones that were associated in CPS-II (EA and ZEBRA). CLL/SLL results from the Women's Health Initiative Observational Study,[18] however, were consistent with CPS-II. De Roos *et al.*[18] reported positive associations for both EA-D and higher levels of EBV DNA with risk of CLL/SLL.

Finally, the antibody immunoblot study[19] also reported stronger findings for CLL/SLL (OR: 2.96, 95%CI: 2.22–3.95). Rollison *et al.*[11] did not observe any heterogeneity by NHL subtype, but CLL/SLL was not included in the study.

Though we did not observe statistically significant ORs for DLBCL, the OR for positive compared to negative EBV serostatus in our elderly population was almost threefold. The most recent WHO classification of lymphoid tumors[25] recognizes EBV+ DLBCL among elderly patients without predisposing immunodeficiency, as a distinct set of tumors. The WHO manual cites age-related immunological deterioration or senescence in immunity as the etiologic factor in these tumors. Our CPS-II results (in an elderly population) suggest that this may extend beyond DLBCL to other types of NHL. It is possible that the role EBV plays in lymphogenesis in immunocompetent individuals is an indirect one, such as chronic immune stimulation, as has been shown for Hepatitis C virus (HCV). HCV binds to receptors on the surface of B- lymphocytes and lowers their threshold for immune response and/or induces DNA mutations.[2]

Though the 2009 IARC Working Group classified EBV as a Group 1 carcinogen for rare types of NHL, the evidence for more common types of NHL was inconclusive. The working group based this conclusion on two cohort studies totaling 115 cases of NHL in apparently immunocompetent individuals, and four case–control studies. In our meta-analysis combining CPS-II results with that from other published studies, there was a statistically significant positive association between EBV antibodies (both EA and VCA) and NHL risk. Our meta-analysis includes five additional studies[6,8,11,18] (including the CPS-II results reported here) that were not part of the IARC report. Four of the five additional studies were prospective (*i.e.*, the blood was collected before NHL diagnosis); and these five studies included more than 1,200 additional cases. Heterogeneity by NHL subtype may have attenuated our results for all NHL and may be one reason for the inconsistent results in individual studies.

A noteworthy strength of this study is the prospective design in the majority of studies included in the meta-analysis. In retrospective studies it cannot be ruled out that the cancer or its treatment may have influenced EBV antibody levels. However, a sensitivity analysis excluding the three traditional case–control studies[7,8,19] showed only a slight attenuation of the EA results and little change to the VCA results. Though we do not have a biologic measure of the immunocompetence of the CPS-II participants, information collected from survey and medical records suggests that these individuals do not have any major immune-crippling conditions such as AIDS or organ transplantation. We do not have any information on autoimmune or inflammatory conditions before blood collection in CPS-II. However, we expect the vast majority of CPS-II participants were unaffected by these disorders as the estimated prevalence in the United States is between 3 and 9%.[26] We expect that, though it may vary from person-to-person for a variety of reasons, immune function in CPS-II is similar to the typical variation in the U.S. population. An additional unknown is whether the timing of the blood collection accurately captured the most important time window for EBV antibody measurement and subsequent NHL risk. Of note, only one blood sample was collected from each participant and they were aged 70 years on average at blood collection. This is about 5 years older than the median age at diagnosis of NHL. It is possible that associations of EBV exposure with risk of NHL may differ in a younger population. In terms of time between blood collection and NHL diagnosis, our sensitivity analysis suggested an association between EBV serostatus and NHL risk only after 3 years of follow-up. However, sample size was extremely limited for this analysis and, though results from some other prospective studies examining this question have been consistent with this finding,[5,9] others have not,[11,18] and it is unclear whether there is a true difference by time since blood collection. An additional limitation of the meta-analysis is that we were unable to include EBNA-1 or ZEBRA as they were not measured consistently (or at all) across other studies. In addition, the varying cutpoints for the antigen levels used by different studies may have attenuated the results of the meta-analysis. Finally, the NHL subtype results must be interpreted with caution given the relatively small sample size.

Evidence from both the meta-analysis and CPS-II suggest that a moderate association between EBV and NHL may extend to more common NHL subtypes like follicular lymphoma and CLL/SLL. Because there may be heterogeneity in the association by subtypes, as well as possible effect modification by organochlorines[7,8,27] or other factors, pooled prospective studies are needed to clarify these possible differences. In addition, tumor blocks and viral DNA may further inform this topic.

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References

1. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Biological agents. Volume 100 B. A review of human carcinogens. *IARC Monogr Eval Carcinog Risks Hum* 2012;100:1–441.
2. Engels EA. Infectious agents as causes of non-Hodgkin lymphoma. *Cancer Epidemiol Biomarkers Prev* 2007;16:401–4.
3. Graham JE, Christian LM, Kiecolt-Glaser JK. Stress, age, and immune function: toward a lifespan approach. *J Behav Med* 2006;29:389–400.
4. Glaser R, Padgett DA, Litsky ML, et al. Stress-associated changes in the steady-state expression of latent Epstein-Barr virus: implications for chronic fatigue syndrome and cancer. *Brain Behav Immun* 2005;19:91–103.
5. Stowe RP, Kozlova EV, Yetman DL, et al. Chronic herpesvirus reactivation occurs in aging. *Exp Gerontol* 2007;42:563–70.
6. Bertrand KA, Birmann BM, Chang ET, et al. A prospective study of Epstein-Barr virus antibodies

and risk of non-Hodgkin lymphoma. *Blood* 2010; 116:3547–53.
7. Hardell E, Eriksson M, Lindstrom G, et al. Case-control study on concentrations of organohalogen compounds and titers of antibodies to Epstein-Barr virus antigens in the etiology of non-Hodgkin lymphoma. *Leuk Lymphoma* 2001;42: 619–29.
8. Hardell K, Carlberg M, Hardell L, et al. Concentrations of organohalogen compounds and titres of antibodies to Epstein-Barr virus antigens and the risk for non-Hodgkin lymphoma. *Oncol Rep* 2009;21:1567–76.
9. Lehtinen T, Lumio J, Dillner J, et al. Increased risk of malignant lymphoma indicated by elevated Epstein-Barr virus antibodies—a prospective study. *Cancer Causes Control* 1993;4:187–93.
10. Mueller N, Mohar A, Evans A, et al. Epstein-Barr virus antibody patterns preceding the diagnosis of non-Hodgkin's lymphoma. *Int J Cancer* 1991;49: 387–93.
11. Rollison DE, Engels EA, Halsey NA, et al. Prediagnostic circulating antibodies to JC and BK human polyomaviruses and risk of non-Hodgkin lymphoma. *Cancer Epidemiol Biomarkers Prev* 2006;15:543–50.
12. Calle EE, Rodriguez C, Jacobs EJ, et al. The American Cancer Society Cancer Prevention Study II Nutrition Cohort: rationale, study design, and baseline characteristics. *Cancer* 2002;94:2490–501.
13. Swerdlow S CE, Harris N. World Health Organization classification of tumours of haematopoietic and lymphoid tissues. Lyon, France: IARC Press, 2008.
14. Brozy J. Development of multiplex serology for HSV-1, HSV-2, EBV and HCMV using recombinant proteins biosciences. Heidelberg: Ruprecht-Karl-University, 2009.
15. Waterboer T, Sehr P, Pawlita M. Suppression of non-specific binding in serological Luminex assays. *J Immunol Methods* 2006;309: 200–4.
16. Waterboer T, Sehr P, Michael KM, et al. Multiplex human papillomavirus serology based on in situ-purified glutathione s-transferase fusion proteins. *Clin Chem* 2005;51:1845–53.
17. Li R, Hertzmark E, Louie M, et al. The SAS LGTPHCURV8 Macro. Available at: http://wwwhsphharvardedu/faculty/spiegelman/lgtphcurv8html, 2006.
18. De Roos AJ, Martinez-Maza O, Jerome KR, et al. Investigation of Epstein-Barr virus as a potential cause of B-Cell Non-Hodgkin Lymphoma in a prospective cohort. *Cancer Epidemiol Biomarkers Prev* 2013:10:1747–55.
19. de Sanjose S, Bosch R, Schouten T, et al. Epstein-Barr virus infection and risk of lymphoma: immunoblot analysis of antibody responses against EBV-related proteins in a large series of lymphoma subjects and matched controls. *Int J Cancer* 2007;121:1806–12.
20. Cochran WG. The combination of estimates from different experiments. *Biometrics* 1954;10: 101–29.
21. Higgins JP, Thompson SG. Quantifying heterogeneity in a meta-analysis. *Stat Med* 2002;21:1539–58.
22. Begg CB, Mazumdar M. Operating characteristics of a rank correlation test for publication bias. *Biometrics* 1994;50:1088–101.
23. Bruu AL, Hjetland R, Holter E, et al. Evaluation of 12 commercial tests for detection of Epstein-Barr virus-specific and heterophile antibodies. *Clin Diagn Lab Immunol* 2000;7:451–6.
24. Miller IG, El-Guindy A. Regulation of Epstein-Barr virus lytic cycle activation in malignant and nonmalignant disease. *J Natl Cancer Inst* 2002;94:1733–5.
25. Swerdlow SH, Campo E, Harris NL, et al, eds. WHO classification of tumours of haematopoietic and lymphoid tissues, Fourth Edition edn. Lyon: International Agency for Research on Cancer, 2008.
26. Cooper GS, Bynum ML, Somers EC. Recent insights in the epidemiology of autoimmune diseases: improved prevalence estimates and understanding of clustering of diseases. *J Autoimmun* 2009;33:197–207.
27. Rothman N, Cantor KP, Blair A, et al. A nested case-control study of non-Hodgkin lymphoma and serum organochlorine residues. *Lancet* 1997; 350:240–4.