Cancer Epidemiology 61 (2019) 79–88

Contents lists available at ScienceDirect

Cancer Epidemiology

journal homepage: www.elsevier.com/locate/canep

Epstein Barr virus antibody reactivity and gastric cancer: A population-based case-control study

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Abbreviations: BMI, body mass index; EA-D, early antigen diffuse; EBNA-1, EBV nuclear antigen-1; EBV, Epstein-Barr virus; GC, gastric carcinoma; HPV, human papillomavirus; *H. pylori*, *Helicobacter pylori*; MFI, median fluorescence intensity; OR, odds ratio; PCR, polymerase chain reaction; SD, standard deviation; VCA-p18, viral capsid antigen; WHO, World Health Organization; ZEBRA, BZLF1-encoded replication activator

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https://doi.org/10.1016/j.canep.2019.05.008
Received 11 January 2019; Received in revised form 14 May 2019; Accepted 17 May 2019
Available online 30 May 2019
1877-7821/

ARTICLE INFO

Keywords:
Gastric cancer
Epstein-Barr virus
Multiplex serology
Causality

ABSTRACT

Background: In contrast to the recognized role of *Helicobacter pylori* in the etiology of non-cardia gastric cancer (GC), there is still insufficient epidemiological evidence for the involvement of Epstein-Barr virus (EBV) in gastric carcinogenesis. We aimed to evaluate the relation of antibody profile and antibody reactivity intensity against four individual EBV proteins to GC risk.

Methods: We used information from 281 GC cases and 2071 age and sex frequency matched controls recruited in the frame of the MCC-Spain multicase-control study, between 2008 and 2013. Sociodemographic, lifestyle and environmental factors were assessed in face-to-face interviews. Antibody responses to four EBV proteins (EBNA-1, ZEBRA, EA-D, and VCA-p18) were analyzed by multiplex serology. Odds ratios (OR) and 95% confidence intervals were calculated by using logistic regression mixed models to evaluate the association of seropositivity and antibody reactivity against EBV proteins with GC, adjusting for GC risk factors. Stratified analyses by tumor location (cardia vs. non-cardia) and morphology (intestinal vs. diffuse) were done.

Results: Among controls, seropositivity for EA-D, ZEBRA, EBNA-1 and VCA-p18 was 85%, 91%, 97% and 99%, respectively. Even though seropositivity for none of the studied proteins was associated with a higher GC risk, increasing antibody reactivity against EBNA-1 and VCA-p18 was associated with higher OR of GC. This association was present for cardia and non-cardia cancer cases, and for intestinal and diffuse types.

Conclusion: Our results support the hypothesis that EBV may play a role in GC etiology, and highlight the importance of evaluating specific antibodies and the dose-response relations when studying widespread infections.

1. Introduction

Gastric carcinoma (GC) is the fifth most common cancer and the third leading cause of cancer-related mortality worldwide [1]. Moreover, GC continues to be one of the malignant tumors with poorest prognosis [2], due to the advanced stage in which generally diagnosis is made.

A model for gastric cancer etiopathogenesis was proposed several decades ago [3,4]. This model has been extended to include new findings that have taken place later on [5–7]. Currently, it is accepted that intestinal GC develops through a series of phases, atrophic gastritis, intestinal metaplasia, dysplasia and adenocarcinoma, and that *Helicobacter pylori* (*H. pylori*) infection is the main risk factor for GC [8], having a very high infection-related attributable fraction (89% of non-cardia GC) [9]. However, among all infected individuals, only a small percentage will eventually develop a malignant neoplasm. Therefore, the role of other causal factors in the etiologic paradigm of GC needs further research.

With much lower attributable fractions, there is sufficient evidence of the role in gastric carcinogenesis of rubber production industry, tobacco smoking, X-radiation and gamma-radiation [10]. Other agents, with limited evidence of gastric carcinogenic effects, are Epstein-Barr virus (EBV), asbestos, inorganic lead compounds, ingested nitrate or nitrite and some dietary components, such as pickled vegetables, salted fish and processed meat [10].

EBV was the first virus identified in a human tumor. In 1997, it was classified as a Group I carcinogen by the International Agency for Research on Cancer [11], and while there is sufficient evidence of its carcinogenic role in humans for several types of lymphoma and for nasopharynx cancer, there is limited evidence of its role in GC [8]. According to a recent systematic review [12], most evidence comes from studies in which EBV was found in the malignant epithelial cells of the gastric tissue, while inconsistent results were reported in studies using serology. In this review [12] only four studies comparing results of EBV serology between GC cases and healthy controls were included, all of them carried out in Eastern Asians or Eastern Asian descendants [13–16]. Only one of these studies [13], in Japan, found a significantly increased odds ratio associated with seropositivity, specifically with VCA (Viral Capsid Antigen) seropositivity [12].

Since EBV infection is so common, EBV seropositivity may not be a good marker for GC risk and a quantitative measure of serological response could be more informative. However, this aspect has been poorly addressed in the available literature. A recent study categorized EBV antibody titer in tertiles, and they reported that increased antibody titers against EBV-VCA were associated with premalignant gastric disease and intestinal-type GC [17]. Kim et al [16] also presented the results of an analysis of tertiles of VCA and EBNA (EBV Nuclear Antigen) antibody titers and found no statistically significant associations with GC. Other authors have compared antibody titers against EBV proteins between patients with EBV-positive GC (EBV DNA present in the tumor) and patients with EBV-negative GC (with no EBV DNA in the tumor) and found increased levels among the former [14,18]. We have attempted to address the limitations of previous studies by investigating both the relation between EBV seropositivity and EBV antibody reactivities, overall and for four viral antigens, with GC risk. For this purpose, we used gastric cancer cases and controls recruited in the frame of the MCC-Spain multicase-control study.

2. Material and methods

MCC-Spain is a multicentric multicase-control study aimed to study environmental and genetic factors involved in the etiology of five different frequent cancers in Spain (gastric, colorectal, breast, prostate and chronic lymphocytic leukemia), which uses a population-based set of controls, randomly selected from the general population living in 12 Spanish provinces: Asturias, Barcelona, Cantabria, Girona, Gipuzkoa, Granada, Huelva, León, Madrid, Murcia, Navarra and Valencia. A total of 459 gastric cancer cases were recruited between 2008 and 2013. In parallel, a set of 4098 population controls was randomly selected from primary care center lists within the areas of the 23 collaborating hospitals. Inclusion criteria required that participants were aged 20–85 years old, had resided for at least 6 months in the catchment areas of the participating hospitals and were able to communicate and answer an epidemiological questionnaire. The study design has been described in detail elsewhere [19]. The study protocol has been reviewed and approved by Ethical Committees of the participating institutions. The study was conducted in accordance with the Declaration of Helsinki of the World Medical Association, and all participants signed a written informed consent.

Cases and controls answered a detailed epidemiological questionnaire, including information on socio-demographic and anthropometric factors, lifestyles, environmental and occupational exposures, medical history, and family history of cancer. The questionnaire is available online at http://www.mccspain.org. Participants were also asked to donate biological samples (blood, urine, nails and hair).

For the present work we excluded cases with tumor located in the upper or middle esophagus or located in the lower esophagus but that were not adenocarcinomas (N = 34), cases not providing a blood

sample for serological analysis (N = 175) or with no valid result from multiplex serology (N = 2), and controls from provinces not recruiting GC cases (N = 486), with history of gastric cancer (N = 6), not providing a blood sample (N = 1263) or not matching any case of the same sex and age-group (N = 272).

Blood samples were processed to serum and aliquoted locally in the first 48 h after collection (maintained in refrigeration in the meantime). Then, they were stored at −80 °C. Samples corresponding to gastric cancer cases and to controls were sent to the Infections and Cancer Epidemiology research group of the German Cancer Research Center (DKFZ), in Heidelberg, for serological analysis. Multiplex serology was used to measure antibody reactivity against four EBV proteins: EBV Nuclear Antigen 1 (EBNA-1), BZLF1-encoded replication activator (ZEBRA), Early Antigen Diffuse (EA-D) and EBV Viral Capsid Antigen (VCA-p18). Additional details about the laboratory technique are described in the Supplementary Appendix.

2.1. Statistical analysis

A descriptive analysis of the association of sociodemographic characteristics and GC risk factors with both, disease status (cases vs. controls) and EBV/antigen specific status in controls (seropositive vs. seronegative), was done by calculating frequency distribution and percentages.

Correlation among the EBV antigens' seroreactivity was assessed using the Spearman Rho statistic.

Multilevel logistic regression mixed models were adjusted to obtain odds ratios for gastric cancer risk in relation to EBV serostatus. All models were adjusted for age (continuous), sex, education (No or incomplete primary school/Primary school/Secondary school/University degree), smoking status (Never smoker/Former smoker/Current smoker), *H. pylori* serostatus (positive/negative), and gastric cancer family history (No GC family history/Only second degree relatives/At least one first degree relative) as fixed effects terms, and geographical area (province) as a random effect term.

Analyses were conducted for overall EBV seropositivity (participants seropositive for two or more proteins) and for antigen-specific seropositivity, using seronegative subjects as the reference group. We further categorized antibody reactivity for each protein in quartiles according to their distribution among controls and repeated the analyses using seroreactivity in quartiles. Logistic regression mixed models were similar to those described above, except for the EBV exposure variable. Tests for linear trends were performed by assigning to each quartile the value of the median value of the Median Fluorescence Intensity (MFI) in that quartile. Additionally we estimated the OR and 95% CI for an increase in one standard deviation in the MFI.

We repeated these analyses to quantify the associations of antigen-specific seroreactivity with cancer risk according to major GC subtypes (by location, cardia and non-cardia cancers; and by morphology, intestinal and diffuse types).

Finally, to evaluate the shape of the dose-response relations between antibody reactivities against the four studied proteins and gastric cancer risk, we used restricted quadratic splines for antibody reactivities with knots at the 5th, 50th, and 95th percentiles of their distribution in the control group. The reference value (odds ratio = 1) was set at the 12.5th percentile of each antibody distribution among controls (586, 3468, 265, and 5475 MFI to ZEBRA, EBNA-1, EA-D, and VCA-p18, respectively). Odds ratios were adjusted by the same variables as the models described above.

3. Results

The study group included 281 GC cases (202 non-cardia, 62 cardia, nine lower third esophageal adenocarcinomas and 8 not classifiable because of overlapping location in cardia and non-cardia or missing subsite information) and 2071controls (Table 1). Cases with adenocarcinoma located in the lower part of the esophagus were maintained in the study group to reduce the risk of excluding cardia GC cases, due to the difficulty of determining the origin (esophageal or gastric) of some tumors located in this area. Sixty-nine percent of cases and 57% of controls were males. Cases were older than controls, had lower education and reported more frequently having family history of GC. Cases also reported more frequently a history of gastritis or heartburn, and were more frequently seropositive for *H. pylori*. There were no differences among cases and controls according to body mass index (BMI), smoking status or familial socioeconomic level at birth.

No differences in the EBV seropositivity rate were observed in controls according to sociodemographic characteristics or GC risk factors. Controls reporting first degree relatives with GC were more frequently ZEBRA seropositive than those without such a family history (Supplementary Table S1).

Correlation among antigen specific seroreactivities, measured as MFI, was moderate between EBNA-1 and VCA-p18 and between ZEBRA and EA-D, and low for the remaining paired combinations (Supplementary Table S2).

Overall seropositivity for EBV, as well as for the four EBV-proteins studied was very high in both, cases and controls (in controls, 99.2% overall, 85.4% for EA-D, 90.6% for ZEBRA, 97.3% for EBNA-1 and 99.5% for VCA-p18) (Table 2). As dichotomous variables, overall seropositivity for EBV and seropositivity for EBNA-1, EA-D and VCA-p18 were not associated with higher GC risk, neither in all cases, nor by anatomic location (cardia and non-cardia) or histologic subtype (intestinal and diffuse). However, seropositivity for ZEBRA was associated with a lower GC risk, mainly with non-cardia located cancer (Table 2).

The distribution of cases and controls by antibody reactivity quartiles and the adjusted ORs for gastric cancer were estimated to assess quantitative serological relations (Table 3). Compared to individuals in the first quartile, individuals in the fourth quartile of antibody reactivity against EBNA-1 and VCA-p18 had an increased gastric cancer risk of 2.12 and 2.19, respectively, with a positive trend for increasing risk across strata. Such associations were not seen with ZEBRA and EA-D. Results were similar by cancer location and morphological subtype, with higher ORs for cardia and for intestinal type (Table 4).

A clear dose-response relation was estimated between antibody reactivity for EBNA-1 and VCA-p18 and gastric cancer risk (Fig. 1). For seroreactivity against EA-D a more subtle trend was also estimated, while for ZEBRA the relation was inverse for low-intermediate levels of seroreactivity and positive thereafter.

4. Discussion

After adjusting for the main known risk factors for GC, our results suggest that EBV may have a role in GC etiology. In this population, reactivity intensity of specific EBV antibodies showed a positive dose-response relation with GC risk. Specifically, GC risk was related to high antibody reactivity for EBNA-1 and VCA-p18 proteins, while no so clear associations were observed for ZEBRA and EA-D. Also, stratified analyses according to GC site and morphology revealed a positive dose-response relationship with both cardia and non-cardia cancer and with diffuse and intestinal subtypes for antibody reactivities for EBNA-1 and VCA-p18. This pattern of antibody response would suggest that GC could be associated with a latent infection more than with a lytic phase, like some authors have reported for nasopharyngeal carcinoma [20].

EBV infection is widespread worldwide, with more than 90% of adult people being seropositive in the majority of populations [11]. Most infections occur early in life, when they are usually asymptomatic, or in young adults, when they can cause infectious mononucleosis. Oral route is thought to be the main mechanism of transmission. After infection, EBV would not be eradicated by the immune system; on the contrary, it persists for life in latently infected B-lymphocytes. According to Pagano, the biological hallmark of all the herpes-group viruses is their phases of infection: acute, latent and reactivated, and

Table 1
Characteristics of the study sample.

Variable	Controls (N = 2071)	GC Cases[a] Cardia (N = 62)	GC Cases[a] Non-cardia (N = 202)	p-value cardia	p-value non-cardia
Sex					
Male	**1183 (57%)**	**54 (87%)**	126 (62%)	**< 0.001**	0.149
Female	**888 (43%)**	**8 (13%)**	76 (38%)		
Age (years)					
< 55	**391 (19%)**	**13 (21%)**	**36 (18%)**	**0.023**	**0.001**
55-64	**492 (24%)**	**17 (27%)**	**30 (15%)**		
65-74	**731 (35%)**	**11 (18%)**	**70 (35%)**		
≥75	**457 (22%)**	**21 (34%)**	**66 (33%)**		
Race					
White/Caucasian	**2036 (98%)**	61 (98%)	**193 (96%)**	0.991	**0.004**
Other	**33 (2%)**	1 (2%)	**9 (4%)**		
Education					
No/incomplete primary school	**427 (21%)**	18 (29%)	**61 (30%)**	0.335	**0.003**
Primary school	**784 (38%)**	24 (39%)	**79 (39%)**		
Secondary school	**528 (25%)**	13 (21%)	**42 (21%)**		
University degree	**332 (16%)**	7 (11%)	**20 (10%)**		
Socioeconomic level at birth					
Low	902 (47%)	33 (53%)	110 (55%)	0.451	0.133
Intermediate	943 (49%)	26 (42%)	85 (42%)		
High	62 (3%)	3 (5%)	6 (3%)		
BMI (Kg/m2)					
< 25	599 (34%)	16 (27%)	66 (35%)	0.410	0.894
25-29.9	816 (46%)	27 (46%)	83 (44%)		
≥30	372 (21%)	16 (27%)	40 (21%)		
Smoking status					
Never smoker	**907 (44%)**	**18 (29%)**	88 (44%)	**0.004**	0.595
Former smoker	**734 (36%)**	**21 (34%)**	67 (33%)		
Current smoker	**421 (20%)**	**23 (37%)**	47 (23%)		
GC family history					
No GC family history	**1817 (88%)**	**50 (82%)**	**155 (78%)**	**0.031**	**< 0.001**
Only 2nd degree relatives	**111 (5%)**	**2 (3%)**	**13 (7%)**		
≥1 first degree relative	**132 (6%)**	**9 (15%)**	**32 (16%)**		
History of gastritis					
No	**1844 (96%)**	56 (92%)	**187 (93%)**	0.063	**0.020**
Yes	**69 (4%)**	5 (8%)	**14 (7%)**		
History of heartburn					
No	**1232 (65%)**	35 (57%)	**103 (52%)**	0.248	**< 0.001**
Yes	**676 (35%)**	26 (43%)	**97 (49%)**		
H. pylori serostatus					
H. pylori-	**240 (12%)**	9 (15%)	**11 (5%)**	0.479	**0.008**
H. pylori+	**1831 (88%)**	53 (85%)	**191 (95%)**		
Histological type					
Adenocarcinoma	–	60 (97%)	186 (92%)		
Other	–	2 (3%)	16 (8%)		
Laurén classification[b]					
Intestinal	–	21 (35%)	81 (44%)		
Diffuse	–	7 (12%)	51 (27%)		
Mixed	–	32 (53%)	9 (5%)		
Not available	–	0	45 (24%)		
WHO classification[b]					
Papillary/Tubular	–	19 (32%)	76 (41%)		
Mucinous	–	2 (3%)	5 (3%)		
Poorly cohesive	–	8 (13%)	54 (29%)		
Mixed	–	1 (2%)	10 (5%)		
Not available	–	30 (50%)	41 (22%)		
Tumor stage					
Localized (TNM stages 0-II)	–	9 (17%)	69 (39%)		
Advanced (TNM stages III-IV)	–	45 (83%)	106 (61%)		
Blood collection moment					
Prior/concomitant to treatment	–	28 (50%)	86 (45%)		
First 2 months after treatment	–	14 (25%)	65 (34%)		
> 2 months after treatment	–	14 (25%)	41 (21%)		

(*continued on next page*)

Table 1 (*continued*)

Variable	Controls (N = 2071)	GC Cases[a] Cardia (N = 62)	GC Cases[a] Non-cardia (N = 202)	p-value cardia	p-value non-cardia
Initial treatment					
Surgery	–	28 (50%)	164 (82%)		
Chemotherapy	–	25 (45%)	36 (18%)		
Chemo-radiotherapy	–	3 (5%)	0		

Sum of cases in some variables does not coincide with overall number of cases because of missing information. Statistically significant differences between cases and controls are highlighted in bold. GC: Gastric cancer; BMI: Body mass index.

[a] Seventeen cases were not included in the table because tumor location could not be classified as cardia or non-cardia.

[b] Only applicable to adenocarcinomas.

most EBV malignancies would be associated with reactivated EBV infection, after a period of latency that may last for decades [21].

Previous studies based on serology have given controversial results regarding EBV having a role in GC. However, current evidence from serological studies is limited by diverse design and analytical shortcomings, such as low sample sizes, absence of a control group, or lack of adjustment for known GC risk factors, like age, sex or smoking. Also, most previous studies have not evaluated the role of antibody titers, then lacking an assessment of dose-response relation. However, it is estimated that EBV can be isolated from approximately 8–9% of gastric tumors, and studies focusing on the presence of the virus in gastric cells have found it to be higher in gastric cancer cells compared to both, the surrounding tissue cells and the gastric tissue from non-cancer subjects. These results support the hypothesis of an etiological role of EBV in the development of GC [8,12,22,23].

In our study, as expected, global seropositivity for EBV was very high (> 99% in both, cases and controls). Among controls, antigen specific seropositivity ranged from 85.4% for EA-D to 99.4% for VCA-p18. These high prevalence figures render difficult the evaluation of the role of EBV in GC etiology based on dichotomized serological results. Then, under the hypothesis that antibody levels could reflect EBV reactivation and could then be a risk marker for GC, we decided to deepen into the evaluation of the dose-response shape between antibody profile and GC risk. For two of the proteins a clear gradient was observed with increasing seroreactivity. Dose-response effect is one of the most cited causality criteria, and is considered to be among those that provide a stronger evidence of a causal relation. Bearing in mind the limitations derived from the case-control design of the study to assess causality, mainly the possibility of reverse causation, we reviewed the fulfillment of the criteria proposed by Sir Austin Bradford Hill in 1965: strength, consistency, specificity, temporality, dose response, plausibility, coherence, experiment and analogy [24].

Strength of the association. According to this criterion, the larger the association between the risk factor and the studied outcome, the more likely to exist a causal relation between them. In our case, increases in antibody reactivity for EBNA-1 and VCA-p18 showed a rise in GC risk of 32% and 41% respectively for one-standard deviation increment, which is a not negligible effect size. In other studies that also assessed EBV infection by serology, the range of ORs has been from 0.3 [0.1–1.3] for VCA IgA to 22.2 [7.8–63.1] for VCA IgG [12,18]. Summary OR from studies that assessed EBV infection by analyzing viral presence in gastric tissue was 10.7 [5.0–22.9] among 29 studies using in situ hybridization and 6.2 [3.1–12.6] among 13 studies using PCR (polymerase chain reaction) [22].

Consistency. This criterion refers to the fact that a single study will never prove a causal relationship: studies from diverse populations should obtain similar findings in order to be able to conclude that a causal relation exists. There are few studies reporting the association of GC with EBV serological status. Most of them found no statistically significant association, and estimated OR were both, under and over unity [12,17,25]. However, differences between the studies, such as in the percentage of seropositivity among controls and in the cut-offs used to define seropositivity, could explain the heterogeneity in their results.

Specificity. This criterion, as formulated by Hill, means that exposures are more likely to be the cause of a disease if that specific factor is only the cause of that specific disease. In the case of EBV, various malignant and non-malignant diseases are accepted to be caused by this virus [8,11]. In our study, by histological subtype and location, although estimated OR were slightly higher for cardia GC and for the intestinal type, the magnitude of the differences are small and confidence intervals overlap, precluding us from considering any specificity by tumor location or histology. Also, as we do not have information about the presence of EBV in tumor cells in our cases, we could not assess whether the associations we found were specific for EBV-positive carcinomas. Nevertheless, the specificity criterion is not considered very relevant nowadays.

Temporality. Modern epidemiology recognizes this criterion as a useful and essential condition when evaluating a causal relationship. Exposure should precede outcome, and it is the work of epidemiologists to guarantee this point or to discuss the limitations of their study designs when coping with this issue. As regards to the natural history of the EBV infection, it is clear that primoinfection occurs, in general, early in life, with nearly 100% of the population being positive to this infection in adulthood. Under this hypothesis, it is not difficult to accept that infection precedes the development of gastric cancer. However, if we hypothesize that reactivation of EBV infection is involved in the carcinogenic process we should also demonstrate that reactivation takes place before the development of cancer, which is not so evident, given that reactivations are usually asymptomatic and antibody levels depend on many factors in addition to the time of the antigenic expression. Our study is a case-control study where exposure (EBV antibody reactivity) was measured at inclusion in the study, which for cases corresponded to the time of diagnosis. Therefore, we cannot establish whether the increased antibody reactivity preceded the disease or appeared after gastric cancer had developed. Immunodepression secondary to the neoplastic disease treatments could also lead to the reactivation of the latent EBV infection. Among our cases, around 31% were in an advanced tumoral stage (TNM stage IV) and 54% had received some treatment (surgery, chemotherapy or radiation therapy) before blood sample was collected. However, stratified analyses by tumoral stage and by treatment status did not show a clear limitation of the observed associations to cases in more advanced stages or in those having started treatment (data not shown). Some of the studies that have analyzed the association between antibodies against EBV and GC risk have measured antibody titers prior to the development of the neoplasm, thus limiting this drawback. Two of them found a positive association with GC, although it was statistically significant only in one of them [14,16], while another one found a non-statistically significant inverse association [15]. The clonal nature of the EBV infecting tumoral epithelial cells observed in GC supports the hypothesis of EBV infection being previous

Table 2
Association between gastric cancer and positivity for antibodies to EBV proteins.

					Tumor location[b]						Tumor histological type[c]					
	Controls[a]	Cases[a]	Gastric cancer (overall)		Non-cardia			Cardia			Intestinal			Diffuse		
	(N = 2052)	(N = 278)	OR (95% CI)[d]	p-value	(N = 200)	OR (95% CI)[d]	p-value	(N = 61)	OR (95% CI)[d]	p-value	(N = 104)	OR (95% CI)[d]	p-value	(N = 59)	OR (95% CI)[d]	p-value
EBV +	2034 (99%)	276 (99%)	0.91 (0.21 - 4.02)	0.898	198 (99%)	0.59 (0.13 - 2.61)	0.484	61 (100%)	[e]		103 (99%)	0.54 (0.07 - 4.34)	0.563	59 (100%)	[e]	
EBV protein:																
EBNA-1 +	1996 (97%)	270 (97%)	0.82 (0.38 - 1.76)	0.610	195 (98%)	0.96 (0.37 - 2.45)	0.925	59 (97%)	0.69 (0.16 - 3.01)	0.624	103 (99%)	3.08 (0.39 - 24.52)	0.288	58 (98%)	1.57 (0.21 - 11.72)	0.658
ZEBRA +	1870 (91%)	241 (87%)	**0.61 (0.41 - 0.89)**	**0.012**	**171 (86%)**	**0.54 (0.35 - 0.82)**	**0.005**	54 (89%)	0.80 (0.35 - 1.82)	0.593	92 (88%)	0.63 (0.33 - 1.20)	0.157	51 (86%)	0.61 (0.28 - 1.32)	0.208
EA-D +	1751 (85%)	238 (86%)	1.00 (0.69 - 1.44)	0.995	168 (84%)	0.88 (0.59 - 1.32)	0.545	55 (90%)	1.55 (0.65 - 3.66)	0.321	90 (87%)	1.02 (0.56 - 1.85)	0.953	53 (90%)	1.54 (0.65 - 3.62)	0.325
VCA-p18 +	2040 (99%)	277 (100%)	1.22 (0.15 - 9.62)	0.851	199 (100%)	0.81 (0.10 - 6.49)	0.847	61 (100%)	[e]		104 (100%)	[e]		59 (100%)	[e]	

Statistically significant associations are highlighted in bold.
[a] Nineteen controls and three gastric cancer cases (two non-cardia and one cardia gastric cancer cases; one intestinal and one diffuse) were not included into the models due to missing values in some of the covariates.
[b] Eight cases with tumor not classifiable as cardia or non-cardia and nine with tumors located in the esophageal lower third were not included in this subgroup analysis.
[c] Ten cases of mixed adenocarcinoma and 106 cases with not Lauren classification information were not included in this subgroup analysis.
[d] Adjusted by age, sex, education, family history of gastric cancer, *H. pylori* infection and smoking status; geographical area included as a random-effect term.
[e] OR estimation not possible due to zero EBV negative or VCA-p18 negative gastric cancer cases in these subgroups.

Table 3
Association between GC and quartiles of antibody reactivities to EBV proteins.

	Controls N (%)	Cases N (%)	OR (95% CI)[a]
EBNA-1:			
< 4382	513 (25%)	48 (17%)	**1.00**[b]
4382- 5725	513 (25%)	54 (19%)	**1.12 (0.74 - 1.70)**
5725- 7198	513 (25%)	73 (26%)	**1.43 (0.97 - 2.12)**
≥ 7198	513 (25%)	103 (37%)	**2.12 (1.46 - 3.08)**
p trend			***< 0.001***
1 SD increase (MFI = 2225)			**1.32 (1.16 – 1.50)**
ZEBRA:			
< 1558	512 (25%)	77 (28%)	1.00[b]
1558- 3000	512 (25%)	56 (20%)	0.73 (0.50 - 1.05)
3000- 4731	516 (25%)	76 (27%)	1.03 (0.73 - 1.46)
≥ 4731	512 (25%)	69 (25%)	0.86 (0.60 - 1.23)
p trend			*0.739*
1 SD increase (MFI = 2345)			1.02 (0.90 – 1.15)
EA-D:			
< 885	513 (25%)	59 (21%)	1.00[b]
885- 2506	516 (25%)	72 (26%)	1.16 (0.80 - 1.68)
2506- 4412	510 (25%)	61 (22%)	1.04 (0.71 - 1.53)
≥ 4412	513 (25%)	86 (31%)	1.41 (0.98 - 2.02)
p trend			*0.089*
1 SD increase (MFI = 2356)			1.12 (0.99 – 1.27)
VCA-p18:			
< 6777	510 (25%)	50 (18%)	**1.00**[b]
6777- 8608	514 (25%)	52 (19%)	**1.02 (0.67 - 1.54)**
8608-10754	517 (25%)	69 (25%)	**1.37 (0.92 - 2.02)**
≥ 10754	511 (25%)	107 (38%)	**2.19 (1.52 - 3.17)**
p trend			***< 0.001***
1 SD increase (MFI = 3084)			**1.41 (1.24 – 1.60)**

Nineteen controls and three gastric cancer cases were not included into the model due to missing values in some of the covariates. Statistically significant associations are highlighted in bold, SD: Standard deviation; MFI: Median Fluorescence Intensity.
[a] Adjusted by age, sex, education, family history of gastric cancer, *H. pylori* infection and smoking status; geographical area included as a random-effect term.
[b] Note that the reference group in the analysis by quartiles is different to the reference group in the analysis of seropositivity vs. seronegativity.

to cancer development [26].

Dose response. Our exploration of the distribution of antibody reactivity against EBNA-1 and VCA-p18 proteins found a significantly increased risk of GC for individuals in the highest quartiles of antigen-specific reactivity. For EA-D the trend was not statistically significant, but a 41% increased risk was estimated for the highest quartile compared to the lowest one. On the other hand, seroreactivity for ZEBRA was related to a lower GC risk, although no trend was observed. Taking into account the lack of a linear trend and that the inverse association observed between ZEBRA seropositivity and gastric cancer was statistically significant only for non-cardia GC in our subgroups analysis, we consider that the lower odds of GC estimated for ZEBRA seropositive participants could be due to chance. Other studies have also assessed the role of antibody titer in the risk of gastric malignant disease development. Levine et al found that antibody titers to EBV VCA, EA-D and EBNA were significantly higher in those that developed an EBV-positive GC than in those developing EBV-negative GC or in controls [14]; Shinkura et al found that titers of VCA-IgA, EA-IgG, and VCA-IgG were significantly higher in the EBV-positive GC cases than in the EBV-negative cases, while no differences were observed in EBNA-IgG titers [18]; Kim et al did not find any statistically significant association between tertiles of antibodies against VCA or EBNA and GC risk, although no statistical test for trend was applied [16]. Cárdenas-Mondragón et al [17] found a significant positive trend for EBV antibody titer and the risk of premalignant gastric lesions and intestinal type GC (but not for the diffuse type).

Table 4
Association between gastric cancer and quartiles of antibody reactivities to EBV proteins, by tumor location and by histological subtype.

		Gastric cancer cases							
		Tumor location[a]				Histological type[b]			
	Controls	Non-cardia		Cardia		Intestinal		Diffuse	
	N (%)	N (%)	OR (95% CI)[c]	N (%)	OR (95% CI)[c]	N (%)	OR (95% CI)[c]	N (%)	OR (95% CI)[c]
EBNA-1:									
< 4382	513 (25%)	36 (18%)	1.00	10 (16%)	1.00	17 (16%)	1.00	12 (20%)	1.00
4382- 5725	513 (25%)	38 (19%)	1.07 (0.66 - 1.72)	12 (20%)	1.14 (0.48 - 2.71)	20 (19%)	1.40 (0.71 - 2.78)	10 (17%)	0.82 (0.35 - 1.92)
5725- 7198	513 (25%)	54 (27%)	1.43 (0.92 - 2.24)	16 (26%)	1.44 (0.64 - 3.26)	27 (26%)	1.70 (0.89 - 3.25)	15 (25%)	1.29 (0.60 - 2.81)
≥ 7198	513 (25%)	72 (36%)	**2.01 (1.32 - 3.08)**	23 (38%)	**2.18 (1.01 - 4.73)**	40 (38%)	**2.90 (1.57 - 5.34)**	22 (37%)	1.90 (0.92 - 3.92)
p trend			***< 0.001***		***0.028***		***< 0.001***		***0.034***
1 SD increase (MFI = 2225)			**1.28 (1.10 – 1.49)**		**1.39 (1.06 – 1.82)**		**1.50 (1.22 – 1.85)**		1.25 (0.96 – 1.63)
ZEBRA:									
< 1558	512 (25%)	53 (27%)	1.00	19 (31%)	1.00	23 (22%)	1.00	17 (29%)	1.00
1558- 3000	512 (25%)	43 (22%)	0.80 (0.52 - 1.23)	11 (18%)	0.62 (0.29 - 1.33)	23 (22%)	0.94 (0.51 - 1.74)	11 (19%)	0.65 (0.30 - 1.41)
3000- 4731	516 (25%)	54 (27%)	1.04 (0.70 - 1.57)	18 (30%)	1.03 (0.53 - 2.01)	36 (35%)	1.70 (0.97 - 2.98)	12 (20%)	0.69 (0.33 - 1.47)
≥ 4731	512 (25%)	50 (25%)	0.90 (0.60 - 1.36)	13 (21%)	0.73 (0.35 - 1.51)	22 (21%)	0.78 (0.42 - 1.46)	19 (32%)	1.17 (0.60 - 2.30)
p trend			*0.854*		*0.593*		*0.678*		*0.517*
1 SD increase (MFI = 2345)			1.03 (0.90 – 1.19)		0.97 (0.75 – 1.26)		1.05 (0.87 – 1.28)		1.10 (0.85 – 1.42)
EA-D:									
< 885	513 (25%)	45 (23%)	1.00	11 (18%)	1.00	18 (17%)	1.00	9 (15%)	1.00
885- 2506	516 (25%)	50 (25%)	1.06 (0.69 - 1.62)	17 (28%)	1.49 (0.68 - 3.24)	30 (29%)	1.53 (0.82 - 2.84)	15 (25%)	1.69 (0.73 - 3.92)
2506- 4412	510 (25%)	41 (21%)	0.91 (0.59 - 1.43)	16 (26%)	1.49 (0.68 - 3.29)	22 (21%)	1.20 (0.62 - 2.31)	15 (25%)	1.69 (0.73 - 3.91)
≥ 4412	513 (25%)	64 (32%)	1.34 (0.89 - 2.01)	17 (28%)	1.66 (0.76 - 3.64)	34 (33%)	1.61 (0.87 - 2.96)	20 (34%)	**2.27 (1.02 - 5.06)**
p trend			*0.182*		*0.258*		*0.245*		*0.057*
1 SD increase (MFI = 2356)			1.12 (0.97 – 1.29)		1.13 (0.88 – 1.47)		1.08 (0.89 – 1.32)		**1.39 (1.09 – 1.76)**
VCA-p18:									
< 6777	510 (25%)	40 (20%)	1.00	9 (15%)	1.00	21 (20%)	1.00	12 (20%)	1.00
6777- 8608	514 (25%)	37 (19%)	0.90 (0.56 - 1.44)	11 (18%)	1.17 (0.48 - 2.89)	19 (18%)	0.92 (0.48 - 1.77)	12 (20%)	1.03 (0.46 - 2.32)
8608-10754	517 (25%)	52 (26%)	1.24 (0.80 - 1.92)	12 (20%)	1.42 (0.58 - 3.45)	26 (25%)	1.29 (0.70 - 2.37)	14 (24%)	1.15 (0.52 - 2.52)
≥ 10754	511 (25%)	71 (36%)	**1.79 (1.18 - 2.72)**	29 (48%)	**3.38 (1.55 - 7.36)**	38 (37%)	**1.97 (1.11 - 3.50)**	21 (36%)	1.94 (0.94 - 4.01)
p trend			***0.001***		***< 0.001***		***0.007***		*0.053*
1 SD increase (MFI = 3084)			**1.32 (1.14 – 1.53)**		**1.57 (1.22 – 2.03)**		**1.37 (1.12 – 1.67)**		**1.46 (1.13 – 1.88)**

Nineteen controls, three GC cases, two non-cardia, one cardia GC cases, one intestinal and one diffuse GC cases were not included into the model due to missing values in some of the covariates. Statistically significant associations are highlighted in bold. SD: Standard deviation.

[a] Eight cases with tumor not classifiable as cardia or non-cardia, nine with tumors located in the esophageal lower third, and 3 with missing information in some of the covariates were not included in this subgroup analysis.

[b] Ten cases of mixed adenocarcinoma, 106 cases with not Laurén classification information, and two with missing information in some of the covariates were not included in this analysis.

[c] Adjusted by age, sex, education, family history of GC, *H. pylori* infection and smoking status; geographical area included as a random-effect term. Note that the reference group in the analyses by quartiles is different to the reference group in the analyses of seropositivity vs. seronegativity.

Plausibility. EBV is a human herpes virus (human herpesvirus 4), for which the International Agency for Research on Cancer considers that there is sufficient evidence to accept its carcinogenicity in the causation of some lymphomas, and of nasopharyngeal carcinoma [8,11]. As regards to EBV's role in GC carcinogenesis, though previous studies have found EBV in about 9% of GC tumors, evidence is still judged insufficient [8]. However, the fact that the viral genome is present in the tumor cell in a monoclonal form, and that transforming EBV proteins are expressed in the tumor cell, provide a mechanistic explanation of how EBV might directly cause at least a proportion of gastric cancers [8]. With respect to the role of serology, studies that have measured both, the presence of EBV in GC tissue samples and serology against the virus have found increased antibody titers among EBV-positive GC [26] and, therefore, serology could be a marker of risk for EBV-positive GC.

Coherence. This criterion means that a causal conclusion should not contradict present substantive knowledge. In our case, since serological studies evaluating EBV infection as a marker of GC risk are still scarce and inconsistent, the results of this work are not in conflict with previous knowledge.

Experiment. According to Hill's criterion, causal interpretations from observational study results would be better supported if randomized trials validated the studied association. There is no doubt about randomized trials being capable of offering the highest level of evidence about a causal relationship, as long as they have been correctly designed and executed. However, in many circumstances, technical or ethical considerations preclude their realization. In our case, given that no therapy is available to treat EBV infection or to avoid infection or reactivations, and that it would be unethical to experimentally expose people to EBV infection, observational studies are the only methodological tool at the epidemiological level to test a possible association with GC. *In vitro, in vivo* or *in silico* experiments can provide mechanistic support to a possible causal link, but a confirmation in epidemiological studies is usually required. Limitations of observational studies should be bear in mind when interpreting their results. In our work, we used different strategies to reduce the risk of the main biases associated with case-control studies [27]. First of all, we randomly recruited controls from the general population, thus reducing the risk of selection bias. We also adjusted all risk estimators to control confounding by matching variables (age, sex and geographical area of residence) and by other GC risk factors.

Analogy. The carcinogenicity of EBV is proved, not only for immune system cell neoplasms but also for an epithelial neoplasm, the carcinoma of the nasopharynx. Also, other viruses have been found to cause carcinomas, like hepatitis B and C viruses causing hepatocarcinoma or HPV (Human Papillomavirus) causing cervical carcinoma. Furthermore, another infectious agent, *H. pylori*, is a recognized etiological agent in non-

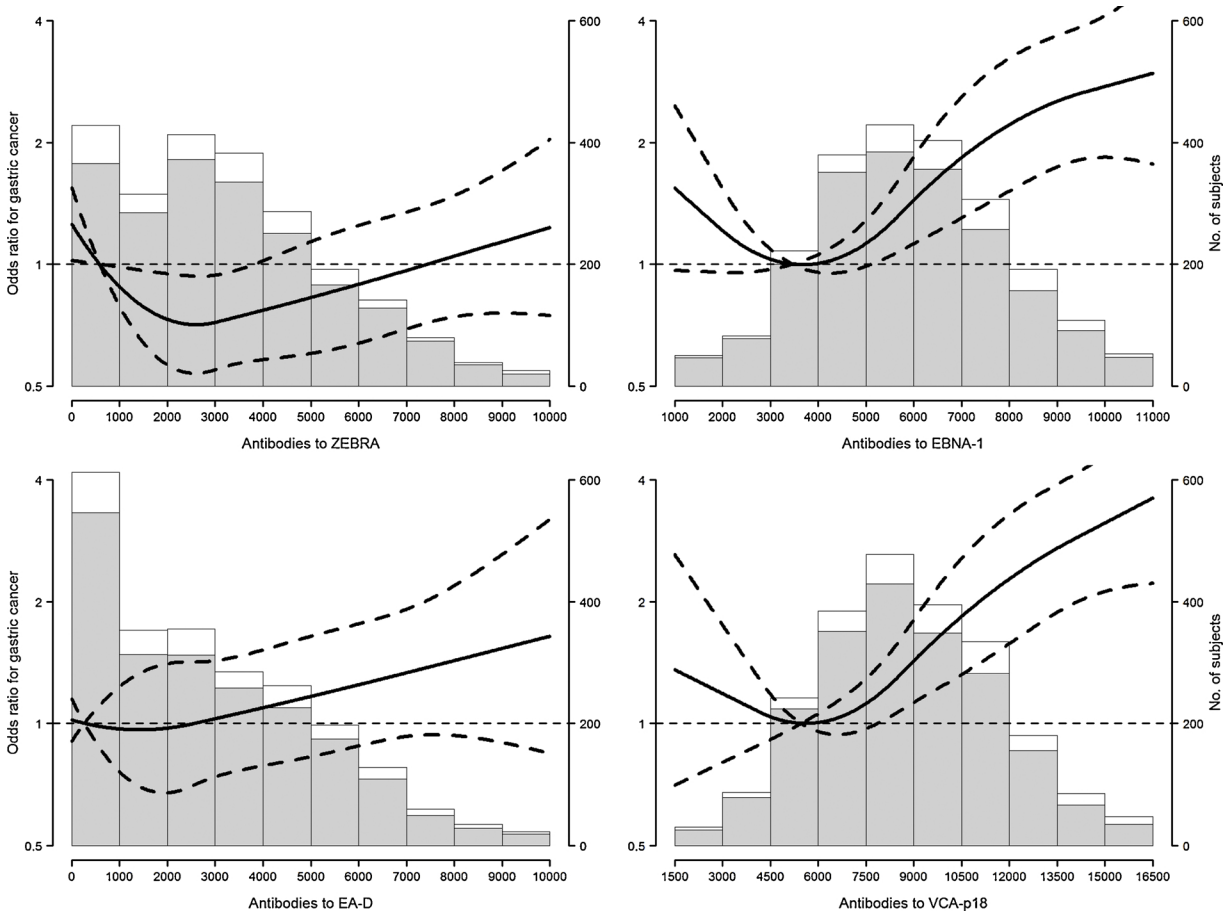

Fig. 1. Odds ratios for gastric cancer by antibody reactivity to Epstein-Barr virus proteins.
Curves represent adjusted odds ratios (solid lines) and their 95% confidence intervals (dashed lines) based on restricted quadratic splines for antibody levels with knots at the 5th, 50th, and 95th percentiles of their control distributions. The reference value (odds ratio = 1) was set at the 12.5th percentile of each antibody distribution among controls (586, 3468, 265, and 5475 median reporter fluorescence intensity to ZEBRA, EBNA-1, EA-D, and VCA-p18, respectively). Odds ratios were adjusted for geographical region (random intercept), age, sex, education, family history of gastric cancer, *H. pylori* infection, and smoking status. Histograms represent each antibody distribution among controls (shaded bars) and gastric cancer cases (white bars).

cardia gastric cancer [8], and although carcinogenic mechanisms of virus and bacteria usually differ, this fact gives support to a possible causal role of EBV infection in GC.

When evaluating our results, several limitations need to be considered. First of all, since this is a case-control study, a possible reverse causation cannot be completely ruled out, in spite of our prospective recruitment of cases. Secondly, our evaluation of EBV seropositivity was based on the multiplex serology, which has not been validated in our population, though this technique has shown high sensitivity and specificity in other settings [28], and allowed the quantification of antibody reactivity against four different EBV proteins. Lastly, we had no information about the presence of EBV in the tumoral cells, precluding us from classifying cases as EBV-positive or EBV-negative, which could have added some insight on the interpretation of our results.

This study has also several strengths. To our knowledge, this is the biggest study to investigate EBV antibody reactivity in GC development in a European population. Controls were randomly selected from the general population and both, cases and controls were residents in different regions across the country, providing a higher representativeness to the studied population. The relatively high sample size, allowed us to perform analyses stratified by tumor location and histological subtype. Also, we gathered comprehensive epidemiological information to adjust all statistical analyses for the main documented confounders, including *H. pylori* seropositivity.

5. Conclusion

The results of this population based case-control study suggest that EBV may play a role in gastric cancer etiopathogenesis. Significantly higher reactivity for EBV EBNA-1 and VCA-p18 proteins was associated with an increased gastric cancer risk. Most of the causality criteria proposed by Bradford Hill are met. Globally, these results suggest that the reactivation of EBV could be an independent risk factor for GC even in a population with a high prevalence of *H. pylori* seropositivity (88.4% among controls). More studies are needed to disentangle the specific role of EBV past infection, reactivation and reinfections as well as *H. pylori* co-infection in GC etiopathology.

Funding

This work was supported by the “Acción Transversal del Cáncer”, approved on the Spanish Ministry Council on the 11th October 2007, Spain; by the CIBER of Epidemiology and Public Health (CIBERESP); by the Instituto de Salud Carlos III, Spain, co-funded by FEDER funds, European Union, -a way to build Europe- [grant numbers PI08/1770, PI09/0773, PI09/1286, PI09/1903, PI09/2078, PI09/1662, PI11/01403, PI14/01219, and PI17/01280]; by the Fundación Marqués de Valdecilla, Spain [grant number API 10/09]; by the Catalan Government DURSI, Spain [grant numbers 2017SGR723, and 2014SGR756]; by the Agència de Gestió d’Ajuts Universitaris i de

Recerca (AGAUR), CERCA Programme / Generalitat de Catalunya for institutional support, Spain [grant number 2017SGR1085]; by the Junta de Castilla y León, Spain [grant number LE22A10-2]; by the Consejería de Salud of the Junta de Andalucía, Spain [grant number 2009-S0143]; and by the Conselleria de Sanitat of the Generalitat Valenciana, Spain [grant number AP061/10].

The funders had not any role in the study design, in the collection, analysis and interpretation of data, in the writing of the report or in the decision to submit the article for publication.

Conflict of interest statement

None declared.

Data statement

Due to confidentiality issues, data used in this manuscript are not publicly available. The anonymized dataset necessary to replicate the analyses would be available upon reasonable request to the MCC-Spain project manager: Gemma Castaño (gemma.castano@isglobal.org).

Authorship contribution statement

Nuria Aragonés: Conception and design, acquisition of data, analysis and interpretation of data, drafting the article, and final approval of the version to be published.

Nerea Fernández de Larrea: Analysis and interpretation of data, drafting the article, and final approval of the version to be published.

Roberto Pastor-Barriuso: Analysis and interpretation of data, revising the article critically for important intellectual content, and final approval of the version to be published.

Angelika Michel: Conception and design, acquisition of data, analysis and interpretation of data, revising the article critically for important intellectual content, and final approval of the version to be published.

Beatriz Romero: Conception and design, acquisition of data, analysis and interpretation of data, revising the article critically for important intellectual content, and final approval of the version to be published.

Michael Pawlita: Conception and design, acquisition of data, analysis and interpretation of data, revising the article critically for important intellectual content, and final approval of the version to be published.

Sara Mayorgas-Torralba: Analysis and interpretation of data, drafting the article, and final approval of the version to be published.

Vicente Martín: Conception and design, revising the article critically for important intellectual content, and final approval of the version to be published.

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Inmaculada Salcedo-Bellido: Analysis and interpretation of data, revising the article critically for important intellectual content, and final approval of the version to be published.

María Dolores Chirlaque: Analysis and interpretation of data, revising the article critically for important intellectual content, and final approval of the version to be published.

Nicole Brenner: Analysis and interpretation of data, revising the article critically for important intellectual content, and final approval of the version to be published.

Manuela Pedraza: Analysis and interpretation of data, revising the article critically for important intellectual content, and final approval of the version to be published.

Xavier Bessa: Acquisition of data, revising the article critically for important intellectual content, and final approval of the version to be published.

Beatriz Pérez-Gómez: Conception and design, acquisition of data, revising the article critically for important intellectual content, and final approval of the version to be published.

Julia Butt: Analysis and interpretation of data, revising the article critically for important intellectual content, and final approval of the version to be published.

Manolis Kogevinas: Conception and design, revising the article critically for important intellectual content, and final approval of the version to be published.

Rosa del Campo: Conception and design, revising the article critically for important intellectual content, and final approval of the version to be published.

Silvia de Sanjosé: Conception and design, revising the article critically for important intellectual content, and final approval of the version to be published.

Tim Waterboer: Analysis and interpretation of data, revising the article critically for important intellectual content, and final approval of the version to be published.

Marina Pollán: Conception and design, drafting the article, and final approval of the version to be published.

Acknowledgments

The authors thank all patients and families for their participation in the MCC-Spain study, as well as all study centers and collaborators.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.canep.2019.05.008.

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