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Epstein-Barr virus and human papillomavirus serum antibodies define the viral status of nasopharyngeal carcinoma in a low endemic country

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Abstract

Epstein-Barr virus (EBV) causes nasopharyngeal carcinoma (NPC) in endemic regions, where almost every tumor is EBV-positive. In Western populations, NPC is rare, and human papillomavirus infection (HPV) has been suggested as another viral cause. We validated multiplex serology with molecular tumor markers, to define EBV-positive, HPV-positive and EBV-/HPV-negative NPCs in the United Kingdom, and analyzed survival differences between those groups. Sera from NPC cases (n = 98) and age- and sex-matched controls (n = 142) from the Head and Neck 5000 clinical cohort study were analyzed. IgA and IgG serum antibodies against 13 EBV antigens were measured and compared with EBER in situ hybridization (EBER-ISH) data of 41 NPC tumors (29 EBER-ISH positive, 12 negative). IgG antibodies to EBV LF2 correctly diagnosed EBV-positive NPCs in 28 of 29 cases, while all EBER-ISH negative NPCs were seronegative to LF2 IgG (specificity = 100%, sensitivity = 97%). HPV early antigen serology was compared to HPV molecular markers (p16 expression, HPV DNA and RNA) available for 41 NPCs (13 positive, 28 negative). Serology matched molecular HPV markers in all but one case (specificity = 100%, sensitivity = 92%). EBV and HPV infections were mutually exclusive. Overall, 67% of the analyzed NPCs were defined as EBV-positive, 18% as HPV-positive and 14% as EBV/HPV-negative. There was no statistical evidence of a difference in survival between the three groups. These data provide evidence that both, EBV-positive and HPV-positive NPCs are present in a low incidence country, and that EBV and HPV serum antibodies correlate with the viral status of the tumor.

KEYWORDS

nasopharyngeal carcinoma, multiplex serology, Epstein-Barr virus, human papillomavirus

Abbreviations: CI, confidence interval; EA-D, early antigen diffuse; EBER-1, Epstein-Barr virus small RNA; EBNA1, EBV nuclear antigen 1; EBV, Epstein-Barr virus; HPV, human papillomavirus; HPyV, human polyomavirus; IHC, immunohistochemistry; IMD, index of multiple deprivation; ISH, in situ hybridization; LSCC, laryngeal squamous cell carcinoma; MFI, median fluorescence intensity; MPG, multiplex papillomavirus genotyping; NCT, National Center for Tumor Diseases; NIHR, National Institute for Health Research; NPC, nasopharyngeal carcinoma; OPC, oropharyngeal carcinoma; ROC, receiver operating characteristic; VCA, viral capsid antigen.

1 | INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a squamous cell cancer arising from the lining of the nasopharynx. The incidence of nasopharyngeal cancer varies widely across the world. Globally, the incidence rate is below one per 100 000 person-years.[1] However, incidence rates in NPC endemic regions, including Southeast Asia, North Africa, China and the Arctic, are up to 30 times higher.[2]

In high-incidence or endemic regions, Epstein-Barr-virus (EBV) is uniformly associated with NPC development.[3] Elevated antibody levels to EBV proteins in NPC patients have been described since 1966[3] in many case-control[4-6] and prospective studies[7,8] and are a useful tool for diagnosis and disease prediction in endemic populations. Until recently, these serological analyses were limited to IgA antibody levels against the viral capsid antigen (VCA), EBV nuclear antigen 1 (EBNA1) and early antigen diffuse (EA-D). In 2018, Coghill et al described a novel EBV antibody risk stratification signature, consisting of 14 IgA and IgG antibodies against diverse EBV proteins for the prediction of NPC development in Taiwan, a high-incidence region.[9] This model for NPC prediction showed an accuracy of 93% for detecting NPCs, compared with an accuracy of 82% for VCAp18/EBNA1 IgA biomarkers alone ($P < .01$).

In low-incidence regions, other risk factors besides EBV infection have been described to be associated with NPC development.[10] The role of smoking as a risk factor has been examined in several studies in both, low incidence and endemic regions, and the risk associated with smoking has been shown to be higher in low incidence countries.[11] More recently, several studies also described human papillomavirus (HPV) as a risk factor for NPCs in low incidence regions, although the causal link remains under debate.[12-15]

HPV, especially type 16, is established as a causal agent for the development of oropharyngeal carcinoma (OPC),[16,17] while the prevalence of HPV-driven head and neck tumors outside the oropharynx is low.[18] However, NPCs and OPCs share properties that distinguish them from other head and neck cancers, like their predominantly viral etiology, early age at disease onset, and similar clinical characteristics, especially early lymph node involvement.[19] In OPCs, early antigen HPV serology (especially antibodies against the HPV16 oncoprotein E6) has been shown to be very strongly associated with molecularly defined HPV-positive OPCs, both at diagnosis and prospectively.[17,20,21] HPV detection in NPCs has been limited to p16 immunohistochemistry (IHC) and HPV DNA detection by PCR or in situ hybridization (ISH).[12-15] Currently, no serological assay has been validated for detecting HPV-positive NPCs.

The aim of our study was thus to examine whether EBV and HPV serum antibodies or antibody pattern can differentiate NPCs associated with EBV, with HPV, and those not associated with either virus, and to describe the roles of EBV and HPV infection in the development of NPCs in a low incidence region. These data have the potential to make important contributions to the clinical management of NPCs. While screening approaches are not reasonable in the United Kingdom, due to low NPC incidence, the viral status of the tumor could reveal potential survival differences and underline the need for specific treatment regimens. HPV-driven OPCs have been shown to have a much better survival than HPV-negative OPCs, and can be identified by HPV16-specific antibodies.[22] Existing analyses of survival of EBV-positive, HPV-positive and EBV/HPV-negative NPCs are sparse and contradictory[13,23] and need to be further investigated.

To this end, we examined the molecular and serological EBV and HPV status of NPC cases from the United Kingdom, a country with low NPC incidence (0.27 per 100 000[24]). The analysis was based on the Head and Neck 5000 clinical cohort study,[25] and included 98 incident NPC cases and 142 age- and sex-matched laryngeal squamous cell carcinoma cases (LSCCs) as controls. We used molecular analyses to validate serology for both, EBV and HPV, and calculated sensitivities and specificities for NPC diagnosis. Moreover, we defined three groups, NPCs associated with either EBV infection or HPV infection, and EBV/HPV-negative NPCs, and compared risk factors, histological subtypes and survival.

What's new?

While Epstein-Barr virus (EBV) causes almost all nasopharyngeal carcinoma (NPC) in endemic regions, human papillomavirus (HPV) may also cause NPCs in low-incidence, Western populations. Here, the authors used molecular tumor markers to validate EBV and HPV multiplex serology to define the viral status of NPCs in the United Kingdom. IgG antibodies to the EBV antigen LF2 and HPV early antigen serology were highly specific and sensitive to identify EBV- and HPV-positive NPCs, respectively. The results show that both EBV-positive and HPV-positive NPCs are present in a low-incidence country, and that EBV and HPV serum antibodies correlate with the viral status of the tumor.

2 | MATERIALS AND METHODS

2.1 | Study population

The Head and Neck 5000 clinical cohort study has been described in detail elsewhere.[25,26] Briefly, 5511 individuals with newly diagnosed head and neck cancer were recruited in 76 centers across the United Kingdom between 2011 and 2014.

The current analysis included 98 incident nasopharyngeal carcinoma cases (ICD-O-3 C11) and as controls 142 age- and sex-matched laryngeal squamous cell carcinomas (LSCC; ICD-O-3 C32) for which no EBV-positive cases[27] and <5% HPV-positive cases[18] are expected (Table 1). Extended pathology reports were requested and available for 76 NPC cases. We cross-checked information captured in histopathology reports against clinical data, and anatomical site misclassification was ruled out, to the best of our knowledge, for 87% (66 of 76) of these NPC cases. Routine clinical measures, including EBER-ISH and p16, were extracted from the histopathological reports.

TABLE 1 NPC case and LSCC control group characteristics

	NPC cases (n = 98)	LSCC controls (n = 142)	*P*-value
Mean age (SD)	53 (13)	55 (11)	.42
Sex			.97
Male	75 (77%)	109 (77%)	
Female	23 (23%)	33 (23)%	
Smoking[a]			<.01
Never	21 (33%)	5 (5%)	
Former	30 (47%)	61 (65%)	
Current	13 (20%)	28 (30%)	
Alcohol consumption[a]			.01
Nondrinker	28 (41%)	29 (30%)	
Moderate	18 (26%)	20 (21%)	
Hazardous	21 (31%)	32 (33%)	
Harmful	1 (1%)	16 (16%)	
Socioeconomic status[a]			.59
1—least deprived	23 (25%)	33 (26%)	
2	19 (21%)	26 (21%)	
3	19 (21%)	32 (25%)	
4	13 (14%)	21 (17%)	
5—most deprived	17 (19%)	14 (11%)	

Abbreviations: LSCC, laryngeal squamous cell carcinoma; NPC, nasopharyngeal carcinoma.
[a]Numbers may not add up to 100% due to missing data.

2.2 | Molecular analysis of tumor tissue

2.2.1 | EBER-ISH and p16 IHC

Epstein-Barr virus small RNA 1 (EBER-1) in situ hybridization (EBER-ISH) is the gold standard for detecting the EBV genome in tumor tissue.[28] EBER-ISH status was available for a total of 41 NPCs. For 34 NPC cases, it was obtained from pathology reports of the clinical centers; for nine NPCs (including two of the above group), formalin-fixed paraffin-embedded (FFPE) tumor tissue was sectioned according to standard protocols[29] and sent for EBER-ISH staining and reading (Leica ISH EBER probe, automated BOND system) to Severn Pathology, an ISO-accredited medical laboratory (Bristol, United Kingdom). p16 immunohistochemistry (IHC) is the clinical gold-standard for assessing HPV status of OPCs. p16 status was available for a total of 41 NPCs, for 20 it was provided by clinical centers and for 22 determined by staining FFPE tissue sections for p16 at the National Center for Tumor Diseases (NCT) tissue bank (Heidelberg, Germany).

2.2.2 | HPV DNA and RNA analysis

HPV molecular analysis included isolation of HPV DNA and RNA according to standard protocols with utmost care to avoid sample cross-contamination.[29]

After DNA and RNA extraction, Multiplex Papillomavirus Genotyping[30,31] (MPG) was used to analyze DNA for the presence of 51 HPV types including all known high-risk HPV types. RT-PCR and hybridization was used to detect HPV type-specific E6*I RNA for the HPV type(s) detected in the MPG assay.[29] Combined HPV DNA and RNA detection is considered the laboratory gold-standard for assessing HPV status of OPCs.[21]

2.2.3 | Histology

Histology data were obtained for 60 NPC cases, either by extracting data from pathology reports (for 37 cases), or by reviewing and classifying the tumor tissue (for 23 cases) by a pathologist.

Cases were categorized into keratinizing NPC (WHO type I), nonkeratinizing differentiated NPC (WHO type II), nonkeratinizing undifferentiated NPC (WHO type III), nonkeratinizing NPC not otherwise specified (WHO type II or III), and basaloid squamous NPC. A total number of 50 cases had an explicit WHO classification of type I, II or III.

2.2.4 | EBV serology

Serological testing of blood taken at diagnosis was performed with multiplex serology, a high-throughput assay for simultaneous detection of serum antibodies against a large number of antigens.[32] Testing for EBV antibodies included separate IgA and IgG detection, as indicated by a recently published NPC risk stratification signature.[9] Sera were preincubated at 1:50 dilution for IgA testing (final dilution 1:100) and at 1:5000 dilution for IgG testing (final dilution 1:10 000) in a serum preincubation buffer based on PBS with 2 mg/mL casein and additionally containing 2 g/L of lysate proteins of *Escherichia coli* overexpressing glutathione-S-transferase (GST)-tag, 5 g/L polyvinyl alcohol and 8 g/L polyvinyl-pyrrolidone.[33]

EBV serology was based on 13 antigens. VCAp18, EBNA1 peptide (pep), EBNA1 truncated (trunc), ZEBRA and EA-D were previously validated for multiplex serology.[34] The remaining eight antigens (BXLF1, LF2, BZLF1, BORF1, BFRF1, BGLF2, BRLF1, BPLF1) derived from an NPC-specific risk stratification signature[9] and were adapted for multiplex serology by recombinant expression as GST-tagged fusion proteins in *E. coli*. The antigens ZEBRA (245 amino acids) and BZLF1 (43 amino acids) display antigenic domains from the same protein, and show an identity of 98% in their 43 overlapping amino acids. For consistency, both, the previously validated ZEBRA and the BZLF1 as part of the NPC-specific risk stratification signature, were included in our assay. Three antigens representing the major capsid protein VP1 of the three human polyomaviruses (HPyV) JC, BK and HPyV6 were included as specificity controls (no association with NPC was expected).

Bound serum antibodies were detected with goat anti-Human IgG-Biotin (1:1000, #109-065-098, Jackson ImmunoResearch, West Grove, Pennsylvania) and goat anti-Human IgA-Biotin (1:1000, #109-065-011, Jackson ImmunoResearch), respectively, and

subsequently stained with streptavidin-R-phycoerythrin (1:750, MOSS Inc., Elk Grove Village, Illinois).

2.2.5 | HPV serology

HPV serology was performed at a final serum dilution of 1:100 with a triple isotype-specific (IgG/IgM/IgA) goat anti-Human antibody as described previously.[32] Analysis included serum antibodies against early (E6 and E7) and late (L1) proteins of high-risk HPV types 16, 18, 31, 33, 35, 45, 52 and 58, and additionally antibodies against early proteins E1, E2 and E4 of HPV16 and HPV18. We defined seropositivity based on the standard definition (HPV16 E6 antibodies >1000 MFI [median fluorescence intensity]) which has been validated for HPV-driven OPCs.[21] In addition, we applied an extended approach previously shown for neck squamous cell carcinoma from unknown primary that includes the standard definition but alternatively allows positivity to three out of four early antigens (E1, E2, E6, E7) for HPV16 and HPV18, or positivity to two early antigens (E6 and E7) for HPV 31, 33, 35, 45, 52 and 58. A serum sample was considered HPV seropositive if either the standard or the extended definition was met. Overall, 12 HPV16-positive cases were defined with the standard definition, and five cases were defined as HPV18-positive using the extended definition.

2.3 | Statistics

Statistical tests for categorical analyses included chi-square test, and Fisher's exact test for small (n < 6) cell counts. *t*-Test was used to compare the mean age of the case and control groups. Differences in MFI values of cases and controls were calculated by Mann-Whitney test.

Smoking was categorized as never, former or current, and alcohol consumption was analyzed in the categories nondrinker, moderate, hazardous or harmful. Detailed information on smoking and alcohol history was obtained at baseline via a self-reported questionnaire as described before.[35] Since there was only one harmful drinker among the NPC cases, the "harmful" and "hazardous" categories were combined in subsequent analyses (Table 1).

Socioeconomic status was categorized in five categories from "1—least deprived" to "5—most deprived" based on the English Index of Multiple Deprivation (IMD) 2010 quintiles using participants' home postcode.[36] This area-based index of deprivation is derived from measures of income, education, crime and barriers to housing.

We used receiver operating characteristic (ROC) analysis of 41 cases with EBER-ISH status to define antigen-specific cut-offs for the EBV antigens based on ≥90% specificity. Resulting cut-off values are listed in Table S1. The technical minimum cut-off above assay background is 30 MFI for IgA at 1:100 dilution and IgG at 1:10 000 dilution, and 50 MFI for the triple isotype-specific (IgG/IgM/IgA) antibody at 1:100 dilution.

Odds ratios for the association of EBV antibodies with NPC were calculated using unconditional logistic regression with 95% confidence intervals (CI) adjusted for age, sex, smoking and alcohol consumption. All NPC cases and controls were included in regression models, and EBV antigens were treated as binary variables, based on the cut-offs described above.

Final EBV status of NPCs was determined if available by (a) EBER-ISH status from FFPE, (b) EBER-ISH data from diagnostic pathology reports, or in the absence of EBER-ISH data, (c) EBV serology. Final HPV status was determined if available by (a) p16 status from FFPE, (b) clinical p16 data, (c) positivity to both HPV DNA and RNA, or in the absence of HPV molecular data, (d) seropositivity as described above.

Overall survival of EBV-positive, HPV-positive and EBV/HPV-negative NPCs, as well as the overall survival stratified by WHO type I, II and II, was plotted in a Kaplan-Meier graph. Cox proportional regression models adjusted for age and sex were used to estimate hazard ratios.

All statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software, Inc., La Jolla, California), Stata 15.0 (StataCorp., College Station, Texas) or SAS enterprise guide 7.1 (SAS Institute, Cary, North Carolina). A *P*-value of .05 was considered as statistically significant.

3 | RESULTS

3.1 | Participant characteristics

The nasopharyngeal carcinoma cases (n = 98) and the laryngeal squamous cell carcinoma cases used as a control group (n = 142) did not differ in mean age, sex and socioeconomic status (Table 1). However, the proportion of never smokers and nondrinkers was significantly higher in NPC cases than among LSCCs (33% vs 5%, $P < .01$, and 41% vs 30%, $P = .01$, respectively).

3.2 | EBV serology validation

All case and control sera were tested for the presence of 26 EBV antibody markers, including IgA and IgG antibodies for 13 antigens. IgA antibody responses to all 13 antigens and IgG antibody responses to all antigens except one (VCAp18) were significantly higher among cases than controls (Figure S1). No significant differences between cases and controls were seen in both IgG and IgA antibody responses to all three human polyomaviruses, with the exception of low-level IgA responses to BK virus (median MFI among controls 306 MFI, median MFI among cases 149 MFI, $P < .01$, compared to 300 and 345 MFI among cases and controls for IgG respectively, $P = .52$). Odds ratios adjusted for age, sex, alcohol and smoking showed strong associations of EBV antibody responses with NPC (Table S2). A higher risk of NPC was observed for all anti-EBV antibodies except for VCAp18 IgG, with odds ratios ranging from 2.7 (95% CI 1.4-5.0) for VCAp18 IgA to 80.5 (95% CI 25.0-258.8) for LF2 IgG and 132.3 (95% CI 17.1-∞) for BGLF2 IgA (Figure 1 and Table S2).

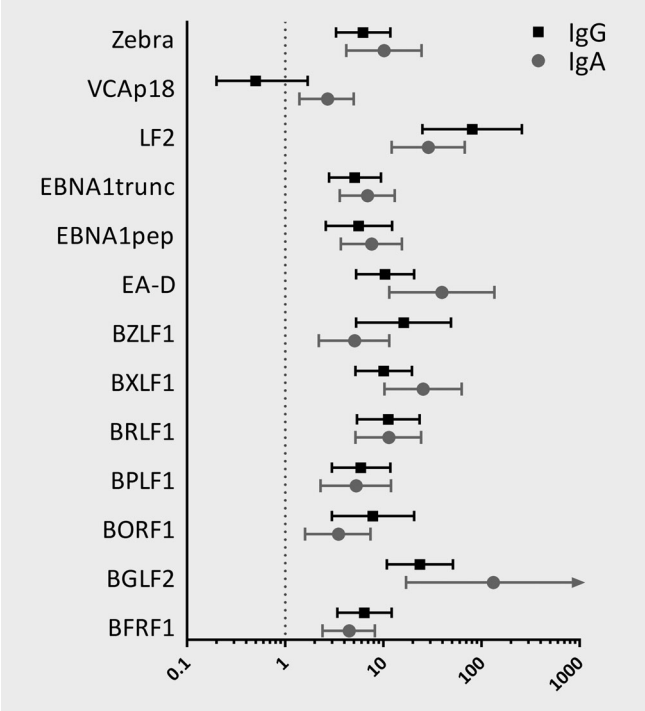

FIGURE 1 Odds ratios (OR) and 95% confidence intervals (CI) adjusted for age, gender, smoking and alcohol consumption for the association of EBV IgA and IgG antibodies with NPC

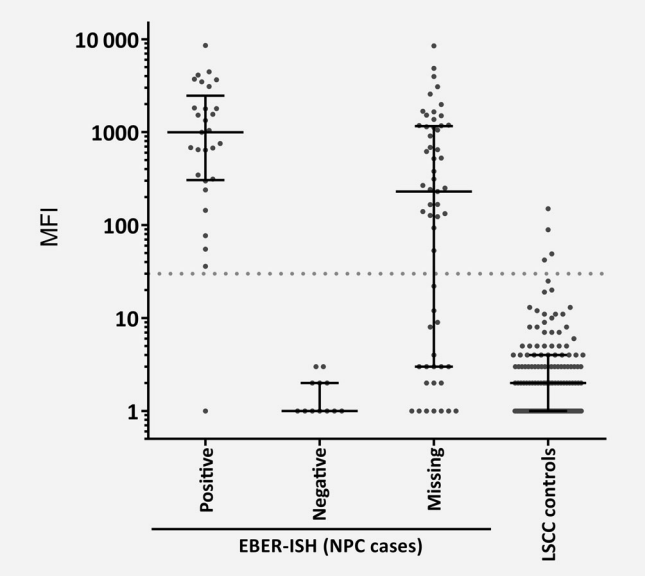

FIGURE 2 Median fluorescence intensities (MFI) for antibody responses against LF2 IgG, stratified by EBER-ISH status for 98 NPC cases (n = 29 positive, n = 12 negative, n = 57 missing) and control laryngeal squamous cell cancer (LSCC; n = 142). The dotted line indicates the seropositivity cut-off (30 MFI) based on receiver operator characteristic (ROC) analysis of NPC cases with available EBER-ISH data

EBV tumor status based on EBER-ISH analysis was available for 41 NPC cases (29 positive, 12 negative). To investigate whether EBV tumor status was reflected by serological markers, IgA and IgG antibodies against 13 EBV proteins were compared to EBER-ISH tumor status for validation. Antigen-specific cut-offs were defined using ROC analysis with EBER-ISH status as gold-standard, and a minimum specificity of 90%. The resulting sensitivities for the 26 combinations of 13 antigens and both IgA and IgG antibodies ranged from 7% for VCAp18 IgG to 97% for BGLF2 IgG and LF2 IgG (Table S1). Comparing IgA and IgG antibody responses, a higher sensitivity of IgG antibodies was observed for 9 out of 13 antigens, whereas IgA antibodies were more sensitive for four antigens; the latter was particularly evident for VCAp18 (66% for IgA, 7% for IgG).

Examining individual antibody performances, LF2 IgG antibodies were able to differentiate EBV-positive NPCs from EBV-negative NPCs with 97% sensitivity and 100% specificity (Figure 2). The sole EBER-ISH positive NPC case that was not identified by LF2 IgG was seronegative for 21 antibody markers and only seropositive to BGLF2 IgA and IgG, EBNA1trunc and EBNA1pep IgG and VCAp18 IgA. Seroprevalence of LF2 IgG in control LSCCs was only 3% (Figure 2). The second and third best-performing antibodies were BGLF2 IgG with a sensitivity of 97% at 92% specificity, and BXLF1

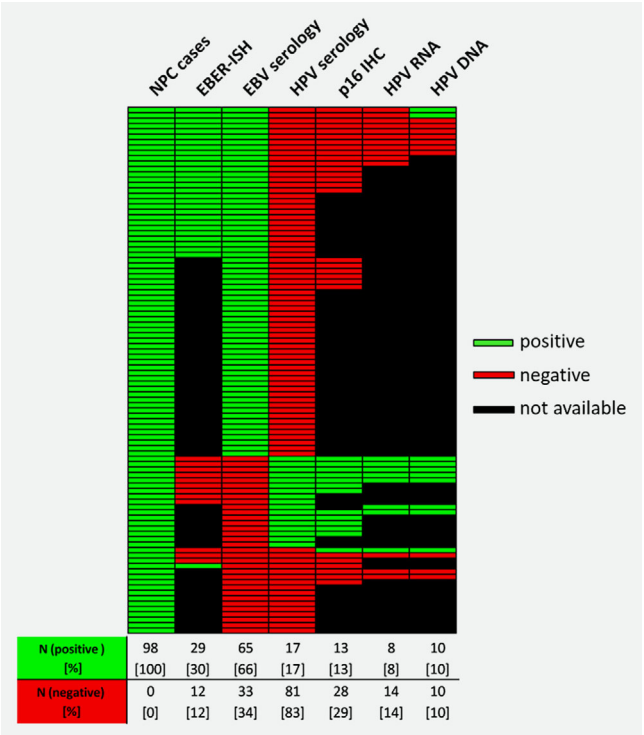

FIGURE 3 Heatmap showing EBV and HPV serostatus as well as molecular markers (EBER-ISH and HPV DNA, HPV RNA and p16 IHC) of all 98 NPC cases. Positivity is shown in green, negativity in red, and black indicates nonavailable data. EBV serology is defined as seropositivity to LF2 IgG antibodies. HPV serology is defined as either HPV16 E6 > 1000 MFI, or positivity to three out of four early antigens of HPV16 or of HPV18, or positivity to two type-concordant early antigens (E6 and E7) of HPV 31, 33, 35, 45, 52 or 58

IgG (93% and 92%, respectively). However, 10% and 17% of the control LSCCs were seropositive for BGLF2 and BXLF1 IgG, respectively.

BGLF2 IgA antibodies showed the strongest association with NPC among all cases (Table S2), based on high specificity (only 1% of control LSCCs were seropositive) but with low sensitivity (only 47% of NPC cases were seropositive; Table S2). Even when restricting the analysis to EBER-ISH-positive cases, the sensitivity of BGLF2 IgA antibodies was much lower (76%) than for BGLF2 IgG and LF2 IgG (both 97%).

Combining several biomarkers did not improve classification of EBV-positive NPCs compared to single markers in this dataset. The combination of the two best stand-alone markers (positivity for at least one of the two), LF2 IgG and BGLF2 IgG, yielded a slightly higher

TABLE 2 Characteristics of EBV-positive, HPV-positive and EBV/HPV-negative NPC cases

	EBV-positive (n = 66)	HPV-positive (n = 18)	EBV/HPV-negative (n = 14)	*P*-value
Mean age (SD)	52 (14)	53 (12)	59 (9)	.19
Sex				.38
Male	49 (74%)	16 (89%)	10 (71%)	
Female	17 (26%)	2 (11%)	4 (29%)	
Stage[a]				.50
I	5 (8%)	0 (0%)	2 (14%)	
II	15 (23%)	4 (22%)	5 (36%)	
III	24 (37%)	8 (44%)	2 (14%)	
IV	21 (32%)	6 (33%)	5 (36%)	
Treatment				.38
Surgery only	1 (2%)	0 (0%)	2 (14%)	
Chemoradiotherapy only	51 (77%)	15 (83%)	9 (64%)	
Radiotherapy only	8 (12%)	3 (17%)	2 (14%)	
Surgery and chemo/radio	3 (5%)	0 (0%)	0 (0%)	
Chemotherapy only	1 (2%)	0 (0%)	0 (0%)	
No treatment	2 (3%)	0 (0%)	1 (7%)	
Smoking[a]				.39
Never	14 (33%)	2 (20%)	5 (45%)	
Former	19 (44%)	5 (50%)	6 (55%)	
Current	10 (23%)	3 (30%)	0 (0%)	
Oral sex partners[a]				.24
1 to 2	10 (50%)	4 (50%)	1 (20%)	
3 to 4	4 (20%)	4 (50%)	2 (40%)	
5 or more	6 (30%)	0 (0%)	2 (40%)	
Alcohol consumption[a]				.10
Nondrinker	21 (48%)	1 (8%)	6 (50%)	
Moderate	12 (27%)	4 (33%)	2 (17%)	
Hazardous/harmful	11 (25%)	7 (58%)	4 (33%)	
Ethnicity				.06
White	52 (79%)	18 (100%)	13 (93%)	
Non-white	14 (21%)	0 (0%)	1 (7%)	
Histology[a]				.0004
WHO type I	0 (0%)	3 (30%)	1 (25%)	
WHO type II	8 (17%)	5 (50%)	3 (75%)	
WHO type III	28 (61%)	2 (20%)	0 (0%)	
WHO type II/III	9 (20%)	0 (0%)	0 (0%)	
Basaloid squamous	1 (2%)	0 (0%)	0 (0%)	

Abbreviations: EBV, Epstein-Barr virus; HPV, human papillomavirus.
[a]Numbers may not add up to 100% due to missing data.

sensitivity of 100% (vs 97% for the best marker LF2 IgG alone) but also a lower specificity of 92% compared to 100% for LF2 IgG alone. Combining LF2 IgG and BGLF2 IgG, the positivity for control LSCCs was 12% and thus higher than for LF2 IgG (3%) or BGLF2 IgG (10%) alone. The gain of sensitivity of the biomarker combination is thus lower than the loss of specificity for both, EBV negative NPCs and control LSCCs in our study.

Based on these data, IgG antibodies to LF2 were the best stand-alone marker for EBV-positive NPCs in the United Kingdom as a non-endemic region within the Head and Neck 5000 study. Thus, LF2 IgG serology was used further to define EBV-positive NPCs by serology in the subsequent analyses described below.

3.3 | HPV serology validation

All case and control sera were tested for the presence of antibodies against high-risk HPV types. No significant differences were observed for HPV antibody levels between cases and controls (data not shown).

HPV tumor status based on p16 IHC analysis was available for 41 NPC cases (13 positive, 28 negative). Nucleic acid analysis results were obtained for 22 (RNA) and 20 (DNA) cases; two cases had invalid DNA but valid RNA results. For all cases with DNA/RNA testing, p16 status was available (Figure 3). All DNA and RNA results matched p16 status, except two cases which were only HPV DNA positive but HPV RNA and p16 negative. Given HPV DNA positivity is insufficient to determine active viral involvement in tumor development,[21] these cases were not considered HPV-driven tumors.

To investigate whether HPV tumor status was reflected by serological markers, antibodies against early and late proteins of high-risk HPV types 16, 18, 31, 33, 35, 45, 52 and 58 were measured and compared to HPV molecular tumor status (as assessed by clinical and laboratory gold-standards p16 and HPV DNA/RNA, respectively) for validation. All p16 negative cases were HPV seronegative (28/28), and all but one (12/13) p16 positive cases were seropositive, resulting in 100% specificity and 92% sensitivity of HPV serology for molecularly defined HPV-positive NPCs (positive for p16, or if p16 was not available, positivity to both HPV DNA and RNA).

Overall, of the 18 HPV positive cases defined by molecular analysis, 13 (72%) were HPV16 positive, four (22%) were HPV18 positive and one (6%) was positive for HPV39. Of the 13 HPV16 positive cases, 12 were also identified as HPV16-positive by serology. The remaining HPV16-positive case (positive for HPV DNA, RNA and p16) was missed by serology. All four HPV18-positive cases based on molecular analysis were also defined as HPV18-positive by serology. The one HPV39-positive case by HPV DNA/RNA was likewise defined as seropositive to HPV18, suggesting antibody cross-reactivity between these two closely related HPV types (HPV39 was not included in the serological analysis). Altogether, serology matched molecular analysis in all except one case (explained by cross-reactivity), and one case was missed by serology.

3.4 | EBV-positive, HPV-positive and EBV/HPV-negative NPCs

Overall, including molecular tumor markers and serology, 67% of the analyzed NPCs were exclusively EBV-positive, 18% were exclusively HPV-positive and 14% were not associated with either of these viral infections (Figure 3).

Comparing the characteristics of EBV-positive, HPV-positive and EBV/HPV-negative NPCs (Table 2) revealed no strong evidence for significant differences among the three groups, despite of histology. Participants with EBV- and HPV-positive NPCs tended to be younger (52 and 53 years vs 59 years for EBV/HPV-negative NPCs, P = .19). The proportion of nondrinkers was lower for HPV-positive NPCs (8%) than for EBV-positive and EBV/HPV-negative NPCs (48% and 50%, respectively, P = .10). There was no current smoker in the group of EBV/HPV-negative NPCs, in contrast to 23% and 30% among EBV-positive and HPV-positive NPCs (P = .39). The histological classification of EBV-positive, HPV-positive and EBV/HPV-negative NPCs was significantly different among the three groups (P = .0004; Figure S2). Of four WHO type I cases, three were HPV-positive (75%), while 28 of 30 WHO type III cases were positive for EBV (93%). Among the 46 EBV-positive NPCs, all but one case with basaloid squamous histology were WHO type II or III cases, that is, there was no EBV-positive WHO type I NPC. Of the 10 HPV-positive NPC, 3 (30%) were WHO type I cases and 7 (70%) were WHO type II or III cases. Among the four EBV/HPV-negative NPC cases with histological data, one (25%) was classified as WHO type I and three (75%) as WHO type II.

All 18 HPV-positive NPCs were identified among white participants, while 14 (21%) of the EBV-positive NPCs were identified among people of color, including 1 Indian, 1 Bangladeshi, 2 Chinese

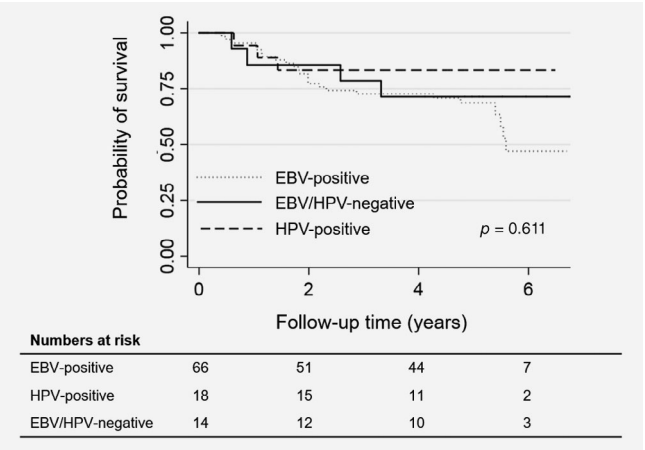

FIGURE 4 Kaplan-Meier plot of all-cause mortality stratified by EBV- and HPV-positive and EBV/HPV-negative NPCs. Survival is not significantly different (P = .61) between EBV-positive (Reference), HPV-positive (hazard ratio [HR] 0.47, 95% confidence interval [CI] 0.13-1.69) and EBV/HPV-negative NPCs (HR 0.91, 95% CI 0.29-2.80)

and 3 with any other Asian background, 1 Caribbean, 2 African and 4 of any other admixture (P = .06).

3.5 | Survival analysis

There was no statistical evidence of a difference in survival between EBV-positive, HPV-positive and EBV/HPV-negative NPCs (P = .61; Figure 4). When compared to EBV-positive NPCs, the hazard ratios were 0.47 (95% CI 0.13-1.69) for HPV-positive NPCs and 0.91 (95% CI 0.29-2.80) for EBV/HPV-negative NPCs.

Comparing survival stratified by WHO histology classification, there was no statistical evidence of a difference in survival between WHO type I, II and III tumors (P = .51; Figure S3).

4 | DISCUSSION

Our analysis of 98 NPC cases from the United Kingdom, a low incidence region, showed that 67% of all NPCs were EBV-positive, 18% were HPV-positive and 14% were not associated with either of these viral infections. This finding is in accordance with the existing, yet sparse literature, showing that 60% to 76% of NPCs in low-incidence regions are EBV-positive and 9% to 16% are HPV-positive.[12-15]

For the first time, we have adapted a previously developed large EBV antigen panel for multiplex serology, and showed that IgG antibodies against LF2 are sufficient to define EBV-positive NPCs. In addition, we provided evidence that the comprehensive HPV multiplex serology panel, established to determine molecular HPV status in OPCs, also reliably identifies HPV tumor status of NPCs.

Our study was based on the Head and Neck 5000 prospective clinical cohort study, including more than 5000 participants with head and neck cancer. Although the number of NPC cases was limited to 98 due to the low incidence of NPCs in this nonendemic region, this likely represents one of the largest NPC case series from a Western country. However, to validate LF2 IgG antibodies as a reliable stand-alone biomarker and to investigate survival differences between EBV/HPV-positive NPCs and those not associated with either viral infection, larger and prospective studies are needed. The inclusion of other markers for defining EBV-positive NPCs, for example, BGLF2 IgG, has to be re-evaluated in other studies with larger case numbers.

One limitation of our study is the use of LSCCs as a control group. Tobacco smoking and alcohol consumption are the largest risk factors for LSCCs, and create an imbalance between cases and controls, which we have adjusted for in our analyses. Since we only used the laryngeal cases to check whether antibodies distinguish between NPCs and non-NPCs, they display a suitable control group, as LSCCs are not known to be associated with EBV infection.[27] LSCC is rarely (<5%)[18] associated with HPV infection, however, we have not calculated risk estimates for HPV serology that could have been affected by HPV-positive LSCCs. Among people with head and neck cancer, LSCCs are the most anatomically different and distant to NPCs, which rules out misclassification bias. There is potential misclassification between LSCCs and OPCs, which however does not affect our analysis, as we expect both, LSCCs and OPCs, to be EBV-negative.

As a strength of our study, we requested extended histopathology reports and cross-checked information between those against the clinical data, to confirm tumor origin and rule out anatomical site misclassification as far as possible, which has been discussed as a potential explanation for HPV-positive NPCs in the past.[14]

The extended antigen panel we used for EBV serology is based on a previous study that described differing IgA and IgG antibody responses in NPC cases and controls from Taiwan, thus reflecting antibody pattern in a high incidence region.[9] All antigens performed well in differentiating NPC cases and controls from the United Kingdom, where NPC incidence is low. Although a lower proportion of NPCs in low incidence regions are associated with EBV infection, the EBV-positive NPCs seem to be broadly serologically similar to NPCs from high incidence regions. However, IgG antibodies to one antigen, LF2, were sufficient to differentiate between EBV-positive and EBV-negative NPCs in our study, rather than a panel of 14 IgA and IgG antibodies.[9] These findings need to be confirmed with larger case numbers. Our cut-off values were based on relatively few cases with available EBER-ISH data. Thus, confidence intervals are wide (LF2 IgG, sensitivity = 96.6% [95% CI 82.2-99.9], specificity = 100% [95% CI 73.5-100.0], Table S1). However, larger case numbers to further evaluate the role of EBV antigens in NPCs are available in endemic countries.

The standard EBV antigens, VCAp18 and EBNA1, both needed very high cut-off values to differentiate between EBV infected individuals and NPC cases, since they are not only markers for NPC, but also for EBV infection, and present in almost every EBV-infected individual. However, we used the full-length VCA-p18 as an antigen. The most EBV-specific antigenic domain comprises the C-terminal amino acids, while the N-terminus shows high homology with other herpes viruses.[37,38] We cannot exclude a C-terminal VCA-p18 antigen may have resulted in higher specificities, however, a study by Fachiroh et al examining IgA antibodies against the C-terminal VCA-p18 peptide for NPC diagnosis showed lower sensitivity and specificity compared to the newly described antibodies in this report.[39] In contrast to VCA-p18 and EBNA1 IgA antibodies, both IgA and IgG antibodies to LF2 seem to be almost always present in EBER-ISH positive NPC patients, and absent in control LSCCs and EBER-ISH-negative NPCs (Figure 2). This clear distinction makes LF2 robust and potentially attractive as a diagnostic and potentially also prospective biomarker. Other than only differentiating EBV-positive from EBV-negative NPCs, the low seroprevalence of LF2 IgG (3%) in control LSCCs (Figure 2) indicates the high value of this marker for identifying individuals with EBV-positive NPCs, assuming healthy people have similar EBV levels as individuals with LSCC. This could be especially important for screening applications in endemic regions.

Little is known about the biological role of LF2 in NPC development. The EBV protein interaction map shows that LF2 binds exclusively to one other EBV protein, Rta (ie, BRLF1).[40] Rta is one of two transcriptional activators for controlling the switch from latent infection to the lytic replication cycle. LF2 overexpression has been shown

to inhibit Rta by downregulating Rta activation of different early lytic promoters, except its own; as a result, lytic activation is blocked.[41] LF2 RNA was also found to be present in Burkitt lymphoma cell lines during latency and in Burkitt lymphoma tumor biopsies.[42]

In our study, we applied HPV serology of high-risk HPV types 16, 18, 31, 33, 35, 45, 52 and 58 to all NPCs. It is the first time a serological definition is used to characterize HPV-positive NPCs. Validation with molecular tumor markers (p16, HPV DNA and RNA) showed that the previously established HPV serology for OPC detection and prediction[17,20,21,43] can be applied equally for NPCs. Interestingly, all 18 HPV-driven NPCs are from white participants as observed previously in other studies[12,14,44] compared to 21% of EBV-positive NPCs being from people of color, half being from Asian countries. There was no overlap (neither for molecular markers, nor for serology) between EBV- and HPV-positive tumors, suggesting that there is no interaction between the viruses in tumor development.

Unlike OPCs, which are mostly (≥90%) associated with HPV16[45] and rarely with HPV16-related types such as HPV33, we identified 13 (72%) HPV16 positive, four (22%) HPV18 positive and one (6%) HPV39 positive NPCs. This strengthens the hypothesis that HPV-positive NPCs are not, or at least not always, an extension from an oropharyngeal primary, as suggested by Singhi et al[14] and instead can represent a separate tumor entity. Multiple HPV types, also including HPV16, HPV18, HPV39 and additionally HPV59, have been described for HPV-positive NPCs before.[46] However, the question whether HPV truly causes NPC requires replication in bigger studies, and additional analyses to establish causality.

Survival analysis of HPV-positive vs EBV-positive and EBV/HPV-negative NPCs did not reveal survival differences in our study. This might be due to our small case groups. Dogan et al found that HPV-positive NPCs have a similar overall survival to EBV-positive NPCs, while EBV/HPV-negative NPCs have a worse overall survival.[13] Another study by Stenmark et al however described a worse outcome for HPV-positive and EBV/HPV-negative NPCs than for EBV-positive NPCs.[23] HPV-positive NPCs are only observed in low-incidence, rather than endemic regions, which makes it difficult to obtain large numbers of NPCs associated with HPV to evaluate different tumor etiologies and patient prognosis with sufficient statistical power. In contrast, it should be noted that the United Kingdom belongs to the countries with relatively high HPV-prevalence in OPC.[47]

Comparing NPC cases positive for EBV or HPV and EBV/HPV-negative NPCs, there is a significant difference between the histological NPC subtypes. It is well established that nonkeratinizing NPCs (WHO type II/III) are especially frequent in endemic regions, where almost all tumors are EBV-positive, while EBV is absent in WHO type I NPCs.[48] In our study, 55 NPCs (92% of all 60 NPCs with histological classification) are either WHO type II or III. Of those 55 NPCs, 45 are positive for EBV (82%), only seven positive for HPV (13%) and three negative for both EBV and HPV (5%). Of the four WHO type I NPCs, none was positive for EBV. This strengthens the close association of EBV-positive and WHO type II/III NPCs.

As suggested by Lo et al,[49] we provide additional evidence that HPV-positive NPCs are associated with keratinizing (WHO type I) NPCs. Of four keratinizing NPCs, three were positive for HPV, and the remaining one was negative for both EBV and HPV. Keratinizing NPCs have also been associated with smoking and alcohol use, a shared characteristic with other head and neck squamous cell carcinomas.[49] Of the four keratinizing NPCs we report, two are former smokers and reported hazardous alcohol consumption; one never smoked, but reported hazardous alcohol consumption; and one was a former smoker, but nondrinker; in summary, tobacco and alcohol exposure seems very high in this population. Comparing the survival of the WHO type I, II and III cases, we did not observe a difference in survival between these histological subtypes (Figure S3). Larger studies are needed for a reliable comparison of survival data.

There is no statistical evidence for a difference in smoking between EBV-positive, HPV-positive and EBV/HPV-negative NPCs. However, it is noticeable that there is no current smoker in the EBV/HPV-negative NPC group. Since EBV infection and smoking have been described as independent risk factors,[50] we expected more current or former smokers in the group of EBV/HPV-negative NPCs. Instead, we observed 55% of former smokers in this group (compared to 50% for HPV-positive and 44% for EBV-positive NPC) and not a single current smoker, while 23% of EBV-positive and 30% of HPV-positive NPC cases were smoking at the time of diagnosis. In consequence, NPCs which are neither associated with EBV, HPV and smoking may be caused by other risk factors, which may include other viral infections not considered in our study. The absence of any smoker in the group of EBV/HPV-negative NPCs could also be based on reporting bias. However, in our study, data on smoking is incomplete and our case groups are too small to draw final conclusions.

In summary, both EBV and HPV serology were included in our analysis and validated with molecular tumor markers, and we have shown that EBV and HPV serum antibodies correlate with the viral status of NPC tumors. Individual serum antibodies or antibody patterns represent an attractive, little invasive diagnostic marker that does not require tumor tissue. The methods we presented here should be applied in further case/control and prospective studies to confirm results with larger case numbers (especially for HPV-positive NPCs), healthy control groups, and prospectively collected serum samples.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

DATA ACCESSIBILITY

Head and Neck 5000 is run as a resource to be used by the research community. Access to the resource can be requested on the study website (http://www.headandneck5000.org.uk/).

ETHICAL STATEMENT

The Head & Neck 5000 study was approved by Frenchay Hospital Research Ethics Committee, reference 10/H0107/57. All participants gave their written informed consent prior to study inclusion.

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SUPPORTING INFORMATION

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