



Research paper

Characterization of human papillomavirus (HPV) 16 E6 seropositive individuals without HPV-associated malignancies after 10 years of follow-up in the UK Biobank

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ABSTRACT

Background: Antibodies against the HPV16 oncoprotein E6 are promising biomarkers for HPV16-driven oropharyngeal cancer (HPV16-OPC) due to their high sensitivity and specificity, and prospective manifestation. In previous studies, 0.7% of controls without HPV-associated malignancies were HPV16 E6 seropositive of which only a minority is expected to develop HPV16-driven cancer. We aimed to characterise HPV16 E6 antibodies in individuals without HPV-associated malignancies.

Methods: We analysed serum antibodies against HPV16 E6, E7, L1 and HPV18 L1 in a random sample ($n = 9,695$) of the prospective UK Biobank cohort (UKB). Excluding individuals with potentially HPV-associated malignancies ($n = 192$), we assessed risk factors for seropositivity by logistic regression.

Findings: In individuals without potentially HPV-associated malignancies ($n = 9,503$), the HPV16 E6 seroprevalence was 0.8%. Seropositivity against HPV16 E6 and all other HPV antigens was strongly associated with sexual behaviour. The seroprevalence of HPV16 E6, L1 and HPV18 L1 increased with the number of lifetime sex partners ($P_{\text{trend}} < 0.005$), and all HPV antibodies were associated with same-sex intercourse ($OR_{E6} 3.1$, 95%CI 1.4–6.9; reference category: no same-sex intercourse). HPV16 E6 and L1 seropositivity were associated with young age (≤ 17 years) at sexual debut ($OR_{E6} 2.0$, 95%CI 1.1–3.7) compared with individuals reporting sexual debut at age ≥ 20 years.

Interpretation: This is the first study characterising HPV16 E6 antibodies in the general UK population. Their strong association with sexual behaviour, and overlapping risk factor profiles with other HPV antibodies support their relevance for HPV16-OPC disease prediction. However, additional risk stratification will be required to identify individuals at highest risk to develop HPV16-OPC.

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Introduction

High-risk mucosal human papillomavirus (HPV) types can cause anogenital and oropharyngeal cancer (OPC). Over the last decades, both the absolute numbers and proportions of OPC attributable to HPV have been dramatically increasing in countries with high human development index. Thus, HPV infection is in the process of replacing

the previously leading risk factors tobacco and alcohol in these countries [1–3]. Based on this trend, in combination with declining incidence rates of cervical cancer due to efficient precursor screening, HPV-driven OPC (HPV-OPC) now poses a greater disease burden than cervical cancer e.g. in the US [4,5]. In the US, UK and Sweden $\geq 70\%$ of OPC are HPV-driven [1,6,7], of which HPV16 accounts for up to 90% (HPV16-OPC) [8].

OPC is three times more frequent in men than in women, and the incidence increases with age, starting at approximately age 45, with a peak between ages 50 and 64 [1,9]. The prophylactic HPV vaccination introduced in 2006 efficiently prevents oral HPV16/18 infection

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Research in context

Evidence before this study Antibodies against the human papillomavirus (HPV) 16 oncoprotein E6 are detectable in almost all patients with HPV16-driven oropharyngeal cancer (HPV16-OPC), sometimes more than 10 years before diagnosis, and are thus promising biomarkers for screening. In previous OPC case-control studies, both with newly diagnosed patients, and based on pre-diagnostic samples collected in prospective cohorts, the HPV16 E6 seroprevalence was on average 0.7% in controls without HPV-associated malignancies. However, based on the rarity of OPC, only a minority of these is expected to truly develop HPV16-OPC. So far, HPV16 E6 antibodies have never been measured and characterized in the potential screening population, i.e. the general population.

Added value of this study Understanding the natural history of HPV16 E6 antibodies in the general population is an important step towards evaluating the feasibility of an HPV16 E6 serology-based HPV16-OPC screening programme as these individuals represent the potential screening population. We characterized HPV16 E6 antibodies in a large sample of the general population aged 40–69 years. We expectedly observed a low HPV16 E6 seroprevalence in individuals without HPV-associated cancer (<1%) confirming previous observations in controls from OPC case-control studies. HPV16 E6 antibodies were strongly associated with sexual behaviour, including increasing lifetime number of sex partners, same-sex intercourse and young age at sexual debut, and with antibodies to other HPV16 proteins. We also estimated the proportion of HPV16 E6 seropositives in the general population expected to develop HPV16-OPC or other HPV16-driven cancers, based on disease incidence rates, lead times (i.e. time between manifestation of antibodies to diagnosis), and analytical sensitivity of the serological assay. Our study suggests that HPV16 E6 antibodies in the general population are exceptional markers of specific HPV16 infections, most likely in the oropharynx, and that they occur more frequently in individuals reporting risky sexual behaviour. Approximately 10% of these individuals are expected to be diagnosed with HPV16-OPC in the future.

Implications of all the available evidence In combination with previous work, our findings support the feasibility of identifying individuals at elevated risk of developing HPV16-OPC with HPV16 E6 antibodies. However, further risk stratification in HPV16 E6 seropositives is needed to implement an efficient HPV16-OPC screening. Additional factors for risk stratification could be demographic factors, such as male gender, sexual behaviour, and presence of additional HPV16 antibodies.

In recent (nested) case-control studies, seropositivity for late (L1) and early (E1, E2, E4, E6, E7) HPV16 proteins was associated with OPC [15,24–26] but HPV16 E6 seropositivity repeatedly indicated the strongest association with odds ratios (OR) >100 [15,24–26]. HPV16 E6 antibodies were detectable up to 28 years prior to OPC diagnosis [15,24,27]. Furthermore, they showed high sensitivity (>90%) and specificity (99%) compared to molecular HPV status of fresh-frozen or formalin-fixed paraffin-embedded OPC tumour tissue defined by presence of HPV16 DNA and/or RNA by in situ hybridization or PCR, and/or p16 immunohistochemistry [20, 28,29,30].

Due to the absence of detectable precursor lesions for OPC, a prognostic biomarker-based serological screening for HPV-OPC would be highly desirable to identify individuals at high risk of developing HPV-OPC. In previous (nested) case-control studies, on average 0.7% of controls without diagnosed HPV-associated malignancies were HPV16 E6 seropositive [15,24,31]. However, only a minority of these individuals is expected to develop HPV-driven cancer. About one percent of HPV16 E6 seropositive individuals were estimated to develop HPV16-OPC annually in the US [24,21]. The controls included in these studies were, however, age-matched to OPC cases and are thus not representative of a putative screening population that would be necessarily below the typical age of clinical diagnosis. Data about HPV16 E6 seropositivity in population-based studies of a to-be-screened population, i.e. >40 years of age is lacking. To evaluate the performance of a hypothetical HPV16 E6 serology-based HPV-OPC screening approach, the natural history of HPV16 E6 antibodies in the general population needs to be investigated.

We characterised HPV16 E6 antibodies in a random subsample ($n = 9695$) of the UK Biobank. UK Biobank is a prospective cohort study comprising approximately 502,000 individuals from the general population of the UK between ages 40 and 69 recruited from 2006 to 2010 [32]. We analysed i) HPV16 E6 seroprevalence and ii) risk factors for HPV16 E6 seropositivity in individuals without HPV-associated malignancies stringently excluding individuals with diagnosed potentially HPV-associated malignancies. In the risk factor analysis, we included a broad range of demographic factors and known or suspected risk factors for HPV16 infection and/or OPC development. All analyses were also performed for antibodies against the HPV16 oncoprotein E7, and the HPV16 L1 capsid protein. In addition, we analysed L1 antibodies against HPV18, a high-risk HPV type known to cause a substantial proportion of cervical but not oropharyngeal cancers [33, 34, 35].

Materials and methods

Study population

The full study population consisted of a randomly drawn subset of 9695 individuals from the UK Biobank cohort [32,36]. UK Biobank obtained ethics approval from the Research Ethics Committee (REC; approval number: 11/NW/0382) and informed consent from all participants enrolled. Among the subset of 9695 individuals were 192 individuals with diagnosed prevalent or incident potentially HPV-associated malignancies (see Supplementary Material 1). These participants were excluded from the main analyses resulting in a final study population of 9503 individuals without diagnosed potentially HPV-associated malignancies further referred to as "UKB study population" (Table 1).

The UK Biobank cohort has been described in detail elsewhere [32]. In brief, between 2006 and 2010, about 502,000 individuals were enrolled at 22 study centres in England, Wales and Scotland. Individuals aged between 40 and 69, and registered with the National Health Service were invited to the study. Upon recruitment, participants provided detailed information on lifestyle, environment and medical history via a touchscreen questionnaire and a computer-assisted verbal

[10,11] and is expected to be efficacious against HPV-OPC. However, in many countries, it is recommended for early adolescent girls only [12,13], and vaccination coverage is low [14]. Thus, HPV vaccination will probably not have a major impact on HPV-OPC incidence for several decades.

In the absence of identifiable precursor lesions, early detection of OPC is difficult and hence cancers are often diagnosed at a late stage. However, although survival of OPC caused by HPV is much higher than that caused by alcohol/tobacco [15–20], morbidity of treatment is severe. Screening tools enabling early detection of HPV-OPC could decrease the morbidity following treatment and further improve survival by e.g. less invasive robotic surgery, preventive tonsillectomy, or therapeutic HPV vaccines [21]. There are several approaches under evaluation for their potential use in secondary prevention of HPV-OPC including detection of oral HPV DNA and serology [21–23].

Table 1
Characteristics of the UKB study population (n = 9503).

variable	category	n	%	
gender	female	5273	55•5	
	male	4230	44•5	
age [years]	40–49	2232	23•5	
	50–59	3109	32•7	
	60–70	4162	43•8	
	NA	8956	94•2	
ethnicity	White	197	2•1	
	Asian	138	1•5	
	Black	37	0•4	
	Chinese	52	0•5	
	Mixed	80	0•8	
	Other	43	0•5	
smoking status	never	5300	55•8	
	former	3227	34•0	
	current	923	9•7	
	NA	53	0•6	
drinking status	current	8732	91•9	
	former	333	3•5	
	never	420	4•4	
	NA	18	0•2	
Townsend deprivation Index ¹	high	3153	33•2	
	medium	3162	33•3	
	low	3180	33•5	
	NA	8	0•1	
annual household income before taxes [1000 £]	< 18	1813	19•1	
	18–30.9	2079	21•9	
	31–51.9	2128	22•4	
	52–100	1644	17•3	
	> 100	441	4•6	
	NA	1398	14•7	
education level ²	1 (highest)	4488	47•2	
	2	1163	12•2	
	3	516	5•4	
	4	1259	13•2	
	5 (lowest)	353	3•7	
	NA	1724	18•1	
lifetime number of sexual partners ³ (LSP) [n]	0	89	0•9	
	1	2321	24•4	
	2–3	1866	19•6	
	4–5	1268	13•3	
	6–10	1356	14•3	
	≥11	976	10•3	
	NA	1627	17•1	
	≥20	2362	24•9	
age at sexual debut ^{1,3} [years] (ASD)	18–19	2907	30•6	
	≤17	3042	32•0	
	never had sex	89	0•9	
	NA	1103	11•6	
	same-sex intercourse ever ^{3,4} (sameSI)	no	8280	87•1
		yes	287	3•0
NA		936	9•8	

¹ categorized in tertiles (see methods).

² education level categorized from highest (1) to lowest (5) (see methods).

³ defined by vaginal, oral or anal sexual intercourse.

⁴ sexual intercourse of men with men, and women with women.

NA: not available.

interview. A range of physical measures and biospecimens (saliva, blood and urine) were also collected upon recruitment.

Multiplex serology

Serum samples were analysed at the German Cancer Research center (DKFZ) in Heidelberg using Multiplex Serology. This high-throughput bead-based suspension array allows simultaneous measurement of serum antibodies against multiple pathogens in one reaction vessel per sample and has been described in detail elsewhere [37,38]. In brief, pathogen-specific antigens were bacterially expressed as GST-X-tag fusion proteins in *E.coli* with an N-terminal GST and a C-terminal SV40-tag. Subsequently, each antigen was in situ purified on a distinct polystyrene bead set (carboxylated MagPlex

Microspheres, Luminex Corp., Austin, Texas, USA) covalently coated with glutathione-casein. Each bead set is filled with two fluorescent dyes at a distinct ratio enabling a Luminex flow cytometer to distinguish between bead sets, i.e. antigens. The included antigen panel has been described by Mentzer et al. [36] and each individual pathogen-specific assay has been previously validated [24,37–48]. In brief, the samples were tested for antibodies to HPV16 (L1, E6, E7), HPV18 (L1), all human herpesviruses, hepatitis viruses B and C, *Toxoplasma gondii*, human T-lymphotropic virus 1, human immunodeficiency virus 1, *Chlamydia trachomatis*, *Helicobacter pylori*, and human polyomaviruses BKV, JCV and MCV. For background determination, one bead set was loaded with GST. The antigen loaded bead sets were mixed and incubated with serum at a final 1:1000 dilution. Subsequently, bound primary serum antibodies were detected with a biotinylated anti-IgG/IgA/IgM secondary antibody and streptavidin-R-phycoerythrin as reporter dye. Per bead set, at least 100 beads were detected in a Luminex 200 flow cytometer and bound serum antibodies were quantified by calculating the median fluorescence intensity (MFI). Net MFI values were calculated by antigen-wise and serum-wise subtraction of background obtained from negative controls and anti-GST measurements, respectively. On each 96-well plate, standard sera were included to assess inter-plate variance. The median coefficient of variation across all plates was 21% (range: 16–26% for all antigens with mean >50MFI).

For the HPV antigens included in this study, standard cut-offs were not previously defined for the 1:1000 serum dilution. Therefore, 778 serum samples from UKB and published studies [49] (including 272 seropositive for HPV16 E6 to increase power) were tested at serum dilutions of 1:100 and 1:1000. Both the previously defined population-based cut-off (HPV16 E6_{pop}) [50] of 484 MFI and the OPC disease-specific cut-off of 1000 MFI (derived from a nested case-control study (HPV16 E6_{dis})) [15] defined for the 1:100 serum dilution were extrapolated to the 1:1000 dilution such that the prevalence in the samples tested at both dilutions remained identical [51]. The following cut-offs were used to determine HPV seropositivity: HPV16 E6_{pop}: 120 MFI, HPV16 E6_{dis}: 240 MFI, HPV16 E7: 150 MFI, HPV16 L1: 175 MFI, HPV18 L1: 175 MFI. The population-based cut-off for E6 antibodies was additionally validated in individuals reporting no sexual partners in the UKB study population as used by Clifford *et al.* to determine the population-based cut-off (484 MFI) at serum dilution 1:100 [50]. This method suggested a very similar population-based cut-off for HPV16 E6 at serum dilution 1:1000 (115 MFI vs. 120 MFI). Given that the above-mentioned serum panel was designed for the extrapolation of the HPV16 E6 cut-off, the prevalence for HPV16 E7, L1 and HPV18 L1 antibodies was low. Thus, cut-offs for HPV16 E7 and the L1 antibodies were determined based on visual inspection of percentile-plots to determine approximate inflection points as described before [52, 53, 54]. Associations between HPV16 E6 seropositivity and coinfections additionally included in the multiplex serology panel are described in Supplementary Table 4.

UKB variable categorization

Covariate information for the UKB study population was obtained from UK Biobank [55]. Most variables including gender, ethnicity, smoking status, drinking status and annual household income before taxes were used as provided by UKB. Ethnic background categories were encoded as follows: “White” (British, Irish, Any other white background), “Mixed” (White and Black Caribbean, White and Black African, White and Asian, Any other mixed background), “Asian” (Indian, Pakistani, Bangladeshi, Any other Asian background), “Black” (Caribbean, African, Any other Black background), “Chinese” (Chinese), “Other” (Other ethnic group) and “NA” (Do not know, Prefer not to answer). For all UKB-specific analyses, age was determined in

years at the time of blood draw, i.e. at recruitment, and categorized in 10-year age intervals (40–49, 50–59, 60–70 years). For the comparison of the UKB study population with Natsal-3 (Supplementary Material 2), age groups were categorized by birth years (1937–1949: $n = 4581$, 1950–1959: $n = 3088$, 1960–1970: $n = 2026$). The questions relating to sexual behaviour are listed in Supplementary Table 5. The following variables were used: lifetime number of sex partners (LSP), same-sex intercourse ever (sameSI) and age at sexual debut (ASD). Sexual intercourse was defined as vaginal, anal and oral intercourse. Variables LSP and ASD include sexual intercourse of the same and

opposite gender while sameSI refers to men having sex with men, and women having sex with women. The categorization of Townsend deprivation index (low: ≤ -3.20 , intermediate: > -3.2 and ≤ -0.75 , high: > -0.75) and age at sexual debut (ASD; low: ≤ 17 years, intermediate: 18–19 years, high: ≥ 20 years) was performed according to tertiles. The education level was derived and ranked according to British qualification levels [56] (high to low): 1: College or University Degree, other professional qualifications such as nursing or teaching; 2: NVQ (National Vocational Qualification), HND (Higher National Diploma), HNC (Higher National Certificate) or

Table 2
Seroprevalences of HPV16 E6, E7, and L1, and HPV18 L1 in the UKB study population.

variable	category	HPV16 E6 _{dis} n (%)	HPV16 E7 n (%)	HPV16 L1 n (%)	HPV18 L1 n (%)
overall		80 (0.8)	293 (3.1)	407 (4.3)	256 (2.7)
gender	female	43 (0.8)	142 (2.7)	294 (5.6)	175 (3.3)
	male	37 (0.9)	151 (3.6)	113 (2.7)	81 (1.9)
age [years]	40–49	17 (0.8)	63 (2.8)	138 (6.2)	84 (3.8)
	50–59	28 (0.9)	88 (2.8)	159 (5.1)	84 (2.7)
	60–70	35 (0.8)	142 (3.4)	110 (2.6)	88 (2.1)
ethnicity	White	78 (0.9)	279 (3.1)	376 (4.2)	223 (2.5)
	Asian	0 (0)	6 (3.0)	5 (2.5)	12 (6.1)
	Black	0 (0)	3 (2.2)	12 (8.7)	9 (6.5)
	Chinese	0 (0)	0 (0)	1 (2.7)	0 (0)
	Mixed	2 (3.8)	0 (0)	5 (9.6)	5 (9.6)
	Other	0 (0)	4 (5.0)	4 (5.0)	5 (6.3)
	NA	0 (0)	1 (2.3)	4 (9.3)	2 (4.7)
smoking status	never	37 (0.7)	151 (2.8)	215 (4.1)	133 (2.5)
	former	35 (1.1)	106 (3.3)	144 (4.5)	89 (2.8)
	current	8 (0.9)	33 (3.6)	46 (5.0)	33 (3.6)
	NA	0 (0)	3 (5.7)	2 (3.8)	1 (1.9)
drinking status	never	0 (0)	17 (4.0)	14 (3.3)	12 (2.9)
	former	3 (0.9)	11 (3.3)	23 (6.9)	10 (3.0)
	current	77 (0.9)	263 (3.0)	369 (4.2)	234 (2.7)
	NA	0 (0)	2 (11.1)	1 (5.6)	0 (0)
Townsend deprivation Index ¹	low	27 (0.8)	91 (2.9)	115 (3.6)	74 (2.3)
	medium	22 (0.7)	87 (2.8)	131 (4.1)	79 (2.5)
	high	30 (1.0)	115 (3.6)	161 (5.1)	102 (3.2)
	NA	1 (12.5)	0 (0)	0 (0)	1 (12.5)
annual household income before taxes ² [1000 £]	< 18	15 (0.8)	51 (2.8)	85 (4.7)	51 (2.8)
	18–30.9	12 (0.6)	68 (3.3)	80 (3.8)	64 (3.1)
	31–51.9	21 (1.0)	61 (2.9)	90 (4.2)	54 (2.5)
	52–100	22 (1.3)	53 (3.2)	77 (4.7)	40 (2.4)
	> 100	2 (0.5)	16 (3.6)	26 (5.9)	12 (2.7)
	NA	8 (0.6)	44 (3.1)	49 (3.5)	35 (2.5)
education level ²	1 (highest)	39 (0.9)	141 (3.1)	200 (4.5)	120 (2.7)
	2	13 (1.1)	33 (2.8)	56 (4.8)	40 (3.4)
	3	0 (0)	13 (2.5)	12 (2.3)	13 (2.5)
	4	11 (0.9)	36 (2.9)	55 (4.4)	28 (2.2)
	5 (lowest)	4 (1.1)	8 (2.3)	16 (4.5)	11 (3.1)
	NA	13 (0.8)	62 (3.6)	68 (3.9)	44 (2.6)
lifetime number of sexual partners ³ (LSP)	0	0 (0)	3 (3.4)	1 (1.1)	0 (0)
	1	9 (0.4)	75 (3.2)	40 (1.7)	41 (1.8)
	2–3	16 (0.9)	52 (2.8)	46 (2.5)	33 (1.8)
	4–5	8 (0.6)	32 (2.5)	73 (5.8)	42 (3.3)
	6–10	19 (1.4)	47 (3.5)	94 (6.9)	49 (3.6)
	11+	15 (1.5)	35 (3.6)	74 (7.6)	39 (4.0)
	NA	13 (0.8)	49 (3.0)	79 (4.9)	52 (3.2)
age at sexual debut ^{1,3} [years] (ASD)	≥ 20	15 (0.6)	80 (3.4)	57 (2.4)	57 (2.4)
	18–19	23 (0.8)	78 (2.7)	127 (4.4)	71 (2.4)
	≤ 17	37 (1.2)	99 (3.3)	191 (6.3)	106 (3.5)
	NA	5 (0.5)	33 (3.0)	31 (2.8)	22 (2.0)
	never had sex	0 (0)	3 (3.4)	1 (1.1)	0 (0)
same-sex intercourse ever ^{3,4} (sameSI)	no	69 (0.8)	247 (3.0)	360 (4.3)	223 (2.7)
	yes	7 (2.4)	17 (5.9)	24 (8.4)	16 (5.6)
	NA	4 (0.4)	29 (3.1)	23 (2.5)	17 (1.8)

Significant seroprevalence difference among non-ordinal variables and p-trend among ordinal variables are displayed in bold font ($p < 0.05$).

In the test for significant trends, the categories "NA" (where applicable) and "never had sex" for age at sexual debut were excluded.

¹ categorized in tertiles.

² education level categorized from highest (1) to lowest (5) (see methods).

³ defined by vaginal, oral or anal sexual intercourse.

⁴ Sexual intercourse of men with men, and women with women

HPV16 E6_{dis}: serostatus determined by disease-specific cut-off

NA: not available.

equivalent; 3: A/AS levels (Advanced / Advanced Subsidiary level: secondary education required for university entrance) or equivalent; 4: GCSE (General Certificate of Secondary Education; taken during secondary education at age of 16 years) / O levels (Ordinary level: replaced by GCSE in 1988) or equivalent; 5: CSE (Certificate of Secondary Education: replaced by GCSE in 1988) or equivalent; and NA: none of the above or question not answered.

Statistical analysis

Pearson's Chi-squared test was used to test for statistical differences among categorical variables. In circumstances where one or more categories comprised five or less individuals, Fisher's exact test was used. Risk factor analysis for HPV seropositivity was performed by univariate and multivariate (adjusted for age and gender if applicable) logistic regression analysis. Estimates for "NA's" were not reported. Statistical significance was considered if $p < 0.05$. As a sensitivity analysis, the risk factor analysis was conducted using the study data set both including and excluding individuals with incident or prevalent potentially HPV-associated malignancies. No substantial differences were observed (not shown). Statistical analysis was performed using R version 3.5.0 (2018–04–23).

Results

The full study cohort comprised a randomly drawn subsample of individuals from the UK Biobank ($n_{total}=9695$). Individuals with prevalent or incident potentially HPV-associated malignancies ($n = 192$) were stringently excluded from the main analysis (see Supplementary Material 1) [32, 36]. HPV antibody patterns in these 192 individuals are described in Supplementary Material 1. The final study population of individuals without HPV-associated malignancies included $n = 9503$ participants and will be further referred to as the "UKB study population". Demographic characteristics of the UKB study population are shown in Table 1. The study population comprised a higher proportion of females (55.9%) than males (44.1%),

was aged between 40 and 70 years (median: 58 years, IQR: 51–64 years) and 94.3% of individuals were of white ethnicity (Table 1).

HPV seroprevalences and risk factors

The HPV16 E6 seroprevalences using the disease-specific cut-off ($E6_{dis}$) was 0.8%. Seroprevalences of the other HPV antigens ranged between 2.7% (HPV18 L1) and 4.3% (HPV16 L1) (Table 2). Crude HPV seroprevalences were evaluated with regard to gender, age, ethnicity, sexual behaviour, smoking, alcohol consumption and different measures of socioeconomic status (Table 2), and a risk factor analysis was conducted using univariate (Supplementary Tables 7–10) and multivariate (Table 3, analyses stratified by gender in Supplementary Tables 11–13) logistic regression analysis. For HPV16 E6, significantly higher seroprevalences were observed for all variables of sexual behaviour, i.e. for an increasing number of lifetime sex partners, lower age at sexual debut and reported history of same-sex intercourse. The odds of HPV16 E6 seropositivity were approximately 4-fold ($E6_{dis}$) elevated for those participants with six or more lifetime sex partners compared with individuals reporting zero to one lifetime sex partners. Individuals reporting their sexual debut at 17 years or earlier had an approximately 2-fold increased risk to be HPV16 E6 seropositive compared to individuals reporting older age (≥ 20 years) at sexual debut (Table 3). This trend was driven exclusively by males (Supplementary Table 11). Participants reporting a history of same-sex intercourse were approximately three times more likely to be HPV16 E6 seropositive than individuals reporting sexual intercourse with the opposite gender only (Table 3). The association with history of same-sex intercourse was observed in males and females (Supplementary Table 13).

Seropositivity against the other measured HPV antigens (HPV16 E7, L1 and HPV18 L1) was also strongly associated with sexual behaviour. HPV L1 antibodies were associated with an increasing number of lifetime sex partners (OR up to 5.2 for HPV16 L1, and up to 2.6 for HPV18 L1), history of same-sex intercourse (about 2-fold for both HPV16 and HPV18 L1) and young age at sexual debut (up to OR 2.3 for HPV16 L1). Interestingly, seropositivity for HPV16 E7 was only

Table 3
Associations of risk factors with seropositivity to HPV16 E6, E7, L1 and HPV18 L1 in the UKB study population. Odds ratios were calculated by logistic regression analysis adjusting for age and gender if applicable. Significant associations ($p < 0.05$) are depicted in bold.

variable (reference)	category ¹	HPV16 E6 _{dis} OR (95% CI)	HPV16 E7OR (95% CI)	HPV16 L1OR (95% CI)	HPV18 L1OR (95% CI)
gender (female)	male	1.1 (0.7–1.7)	1.3 (1.1–1.7)	0.5 (0.4–0.6)	0.6 (0.4–0.8)
age(40–49 years)	50–59 years	1.2 (0.7–2.2)	1.0 (0.7–1.4)	0.8 (0.7–1.0)	0.7 (0.5–1.0)
	60–70 years	1.1 (0.6–2.0)	1.2 (0.9–1.6)	0.4 (0.3–0.5)	0.6 (0.4–0.8)
	LSP ² (0–1)	2–3	2.4 (1.1–5.4)	0.9 (0.6–1.3)	1.4 (0.9–2.1)
LSP ² (0–1)	4–5	1.8 (0.7–4.7)	0.8 (0.5–1.2)	3.4 (2.3–5.0)	1.9 (1.2–3.0)
	6–10	4.2 (1.8–9.3)	1.1 (0.8–1.6)	4.2 (2.9–6.2)	2.2 (1.4–3.3)
	≥ 11	4.7 (2.0–11.2)	1.1 (0.7–1.7)	5.2 (3.5–7.8)	2.6 (1.6–4.1)
	ASD ² (≥ 20 years)	≤ 17 years	2.0 (1.1–3.7)	1.0 (0.7–1.4)	2.3 (1.7–3.1)
ASD ² (≥ 20 years)	18–19 years	1.3 (0.7–2.5)	0.8 (0.6–1.1)	1.6 (1.2–2.3)	0.9 (0.7–1.3)
	sameSI ^{2,3} (no)	yes	3.1 (1.4–6.9)	2.1 (1.2–3.5)	1.9 (1.3–3.0)
smoking status(never)	current	1.2 (0.6–2.7)	1.2 (0.8–1.8)	1.3 (1.0–1.9)	1.5 (1.0–2.3)
	former	1.6 (1.0–2.5)	1.1 (0.9–1.4)	1.3 (1.1–1.6)	1.2 (0.9–1.6)
drinking status(current)	never	not possible*	1.4 (0.9–2.4)	0.7 (0.4–1.2)	1.0 (0.5–1.8)
	former	1.0 (0.3–3.3)	1.1 (0.6–2.1)	1.6 (1.1–2.5)	1.1 (0.6–2.1)

¹ Associations for "NA's" not shown.
² defined by vaginal, oral or anal sexual intercourse.
³ Sexual intercourse of men with men, and women with women
 OR: odds ratio
 CI: confidence interval
 LSP: lifetime number of sex partners (vaginal, oral, anal)
 ASD: age at sexual debut
 sameSI: same-sex intercourse ever
 HPV16 E6_{dis}: serostatus determined by disease-specific cut-off
 *there were no seropositives in this category.

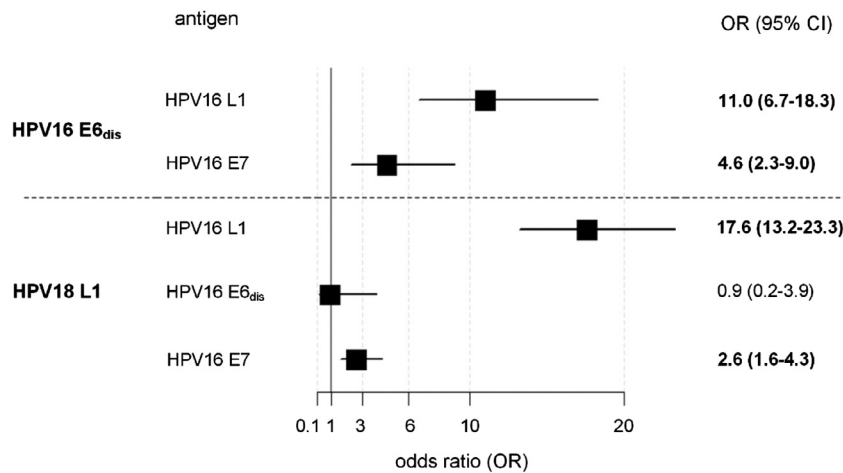


Fig. 1. Associations of seropositivity for HPV16 E6 and HPV18 L1 with seropositivity for other HPV16 antigens. Odds ratios (OR) and corresponding 95% confidence intervals (CI) were determined by logistic regression adjusting for age and gender in the UKB study population. Significant associations ($p < 0.05$) are illustrated in bold. HPV16 E6_{dis}: serostatus determined by disease-specific cut-off.

associated with same-sex intercourse (OR 2•1). No trend of elevated HPV16 E7 seroprevalences with increasing lifetime number of sex partners or younger age at sexual debut were observed.

Antibodies against the HPV16 early antigens E6 and E7 were slightly more frequent in males, as opposed to HPV16 and 18 L1 antibodies which were more prevalent in females (Tables 2 and 3). HPV16 and 18 L1 seroprevalences decreased with increasing age translating into ORs of 0•4 (95% CI 0•3–0•5) and 0•6 (95% CI 0•4–0•8) for the oldest (60–70 years) age group to be seropositive for HPV16 and 18 L1, respectively, versus the youngest age group (40–49 years), while no trend with age was observed for seropositivity to HPV16 E6 or E7 (Tables 2 and 3). No strong or consistent associations with smoking behaviour or alcohol consumption were detected for either HPV16 E6 or the other HPV antibodies (Table 3). As representative measures for socioeconomic status (SES), Townsend deprivation index, household net income and education level were evaluated (Supplementary Table 12). No strong or consistent trends across these measures of SES were observed with HPV seropositivity.

Crude seroprevalences and the risk factor analysis for the population-based cut-off (E6_{pop}) are shown in Supplementary Tables 14 and 15. Associations with the same risk factors but generally lower odds ratios were observed for the population-based versus disease-specific HPV16 E6 cut-off (Supplementary Table 15).

Associations among HPV antibodies

HPV16 E6 seropositive individuals were significantly more frequently seropositive for other HPV16 antigens than HPV16 E6 seronegative participants (Supplementary Table 16). This resulted in strong associations among all pairs of HPV16 antigens (Fig. 1). Seropositivity for HPV16 E6 determined by the population-based cut-off was also highly associated with HPV16 E7 and L1 seropositivity (Supplementary Fig. 4) but the strength of the associations increased when applying the disease-specific cut-off (Fig. 1). In contrast, seropositivity for HPV18 L1 was strongly associated with HPV16 L1 and much weaker with HPV16 E7 antibodies, but not with HPV16 E6 antibodies (Fig. 1, Supplementary Fig. 4).

Representativeness of results: comparison of sexual behaviour with Natsal-3

To assess the representativeness and generalizability of the observed associations of HPV antibodies with sexual behaviour for

the general UK population, the reported sexual behaviour within the UKB study population was compared to the third wave of the National Survey of Sexual Attitudes and Lifestyles (Natsal-3) [57]. Sexual behaviour by gender and birth cohort were very similar in the UKB study population and Natsal-3. The comparison is shown and discussed in Supplementary Material 2.

The risk of an HPV16 E6 seropositive individual to develop an HPV16-driven cancer

Using publicly available data on incidence rates and biomarker characteristics, we estimated the risk of an HPV16 E6 seropositive individual in the UKB study population to develop an HPV16-driven cancer in the future. According to our thought experiment, 9•6% (“credible” interval: 4•1–18•0%) of HPV16 E6 seropositive (E6_{dis}) individuals are expected to develop an HPV16-driven cancer. The majority of these individuals (70%) is expected to develop HPV16-OPC. The rationale, results, assumptions and limitations of this thought experiment are described in Supplementary Material 3.

Discussion

This is the first report evaluating HPV16 E6 antibodies in the general population, by providing population-based seroprevalence estimates, and by conducting a comprehensive risk factor analysis. High sensitivity, specificity and their presence many years before diagnosis make HPV16 E6 antibodies promising biomarkers for secondary prevention of HPV16-OPC [15,21,24,28]. However, despite increasing OPC incidence rates in the general population of many countries, an HPV16 E6 seroprevalence of 0•7%, as suggested by previous (nested) case-control studies [15,24,31], is likely to overestimate the true proportion of future HPV-driven cancer cases (see Supplementary Material 3). Thus, if applied without further risk stratification, an HPV16 E6 serology-based screening scenario would result in a substantial number of false-positives. In a first attempt to characterize HPV16 E6 antibodies in healthy individuals, the few available seropositive controls from several (nested) case-control studies ($n = 32$) were evaluated in a pooled analysis by Lang Kuhs et al. [31]. A limited number of demographic variables such as age, gender, alcohol consumption, smoking status and region of origin was investigated, and no strong or consistent association with HPV16 E6 seropositivity was detected. Importantly, HPV16 E6 antibodies have never been described in a large sample of the general population, i.e. the potential screening population so far.

As this is the first study to investigate HPV16 E6 antibodies in the general population, our understanding of the HPV16 E6 antibody response and its magnitude, i.e. antibody titers or levels, is incomplete. Antibody levels against HPV L1 antigens are considered markers of viral load, and the immune response capabilities of the host [58,59]. In analogy, high HPV16 E6 antibody levels in seropositive individuals without HPV-associated malignancies in the general population might represent high E6 expression, or a high number of HPV-infected cells expressing E6 at sites visible to the (humoral) immune system, such as epithelial cells in the lymphoepithelial tissue of the oropharynx, or HPV-transformed lesions breaking through the basal membrane during anogenital cancer progression. Thus, these individuals might be at higher risk of developing HPV16-OPC, or other HPV16-driven cancers, in the near future [23,24,28]. In contrast, HPV16 E6 seropositive individuals with low E6 antibody levels may harbour HPV16 infections that may or may not develop into precursor lesions or cancer in the future, potentially eliciting higher antibody responses while tumorigenesis progresses.

This is why we investigated HPV16 E6 antibody levels with two cut-offs. With the HPV16 E6 disease-specific cut-off that showed improved specificity for detecting HPV16-OPC without losing sensitivity in a previous OPC case-control study [15], we observed an HPV16 E6 seroprevalence of 0.8% in the UKB study population. This compares well to the average seroprevalence of 0.7% in previous (nested) case-control studies [15,24,31,60] and confirms the rarity of HPV16 E6 seropositivity in the general population. Using a less stringent population-based cut-off derived from HPV16 E6 seroreactivity measured in individuals reporting no previous sex partners [50], a seroprevalence of 1.5% was determined [50,61] (Supplementary Table 14).

The biological plausibility of HPV16 E6 antibodies measured in the general population, as opposed to e.g. mere technical artefacts of the immunoassay, is supported by the observed associations with sexual behaviour (i.e. high numbers of lifetime sex partners, history of same-sex intercourse in both genders, and young age at sexual debut in males), largely overlapping risk factor profiles for the measured HPV antibodies, and strong associations between antibodies against different HPV16 proteins. Nevertheless, our understanding of the biological interpretation of HPV16 E6 seropositivity and antibody levels based on previous case-control studies may need to be refined for HPV16 E6 antibodies observed in the general population. HPV L1 antibodies are generally considered markers of past and present infection, i.e. cumulative exposure markers [58,62–64]. In contrast, antibodies against the HPV16 oncoproteins E6 and E7 are considered disease markers as they have been repeatedly shown to be strongly associated with HPV-driven cancer in (nested) case-control studies on oropharyngeal and anogenital cancer [15,24,40,60,65,66]. However, according to our estimations and those of Kreimer *et al.*, approximately 90% of E6 seropositive individuals in the general population are not expected to develop an HPV16-driven cancer (see Supplementary Material 3 and [21]). This may suggest that HPV16 E6 antibodies are not necessarily strict disease markers in the general population, but represent i) rare markers of – presumably very specific – infections, such as infections at particular sites that allow high viral load replication, or those with distinct molecular profiles (e.g. abortive infections at sites which do not support a productive infection due to the characteristics of the epithelium [67]), or ii) specific host factors, such as individual immunogenetic factors.

Our data indicate that sexual behaviour may play an important role in the distinction between an HPV16 infection which does or does not induce E6 antibodies. It is understood that HPV16 infections differ by anatomical site due to different characteristics of the respective epithelia (e.g. at the cervix and penis). This, in combination with the observed strong associations of HPV16 E6

seropositivity with sexual behaviour, suggests that the induction of HPV16 E6 antibodies correlates with sexual behaviour or practices. In the UKB study population, HPV16 E6 antibodies were associated with all investigated measures of sexual behaviour. However, we were not able to identify a specific sexual behaviour or practice that confers an elevated risk for HPV16 E6 seropositivity as i) the data on sexual behaviour collected in UKB does not allow to sufficiently distinguish between sexual practices (e.g. the number of lifetime sex partners included vaginal, anal and oral sex partners) and ii) same-sex intercourse in both males and females conferred a similarly elevated risk for HPV16 E6 seropositivity (Supplementary Table 13). The increasing risk for HPV16 E6 seropositivity with increasing numbers of lifetime sex partners and younger age at sexual debut in men suggest that a higher level of exposure to HPV16 (e.g. higher viral loads) might facilitate the establishment of an HPV16 infection inducing E6 antibodies.

Genito-oral transmission of HPV16 to the oropharynx might induce an HPV16 infection which elicits E6 antibodies early after infection. HPV-OPC arises from the tonsillar crypts lined with reticulated epithelium in which HPV(16) is not expected to establish a productive infection [67]. In a resulting abortive infection, sufficiently high expression or accumulation of HPV16 E6 may induce an early humoral immune response due to the close proximity to antigen presenting cells and lymphocytes in the lymphatic tissue of the oropharynx [68]. This hypothesis is further supported by the previously observed association of (HPV16-)OPC with the performance of oral sex and a higher number of oral sex partners [69], and manifestation of E6 antibodies up to 28 years before HPV-OPC diagnosis [15, 24, 27]. If HPV16 E6 antibodies were rare markers of an oropharyngeal HPV16 infection, this also suggests that most but not all of these infections are cleared by the innate and adaptive immune system and only a minority unable to mount an effective immune response may develop OPC comparable to what was observed for HPV infections at the cervix [70]. Apparently, these infections induce a B-cell response but no effective cytotoxic T-cell response in those developing HPV-OPC. Studies have shown that cytotoxic T-cells infiltrate HPV-OPC tumour tissue and stroma but are apparently unable to efficiently prevent neoplastic progression [71,72]. This is probably attributable to mechanisms for immune evasion utilized by HPV (reviewed e.g. by [73, 74]) and potentially supported by host genetic variances [75].

Higher OPC incidences in males suggest that performing oral sex on females might confer a higher risk of oropharyngeal HPV16 infection. This could be caused by a potentially more efficient genito-oral transmission from the female genital tract to the male oropharynx due to i) potentially higher viral loads in the female genital tract or ii) existing neutralising antibodies in females elicited by a previous genital infection which prevent a secondary oropharyngeal HPV16 infection. In the UKB study population, the HPV16 E6 seroprevalence was slightly but not significantly higher in males than females. However, this does not reflect gender-specific OPC incidence rates which are three times higher in males than females [1,9]. Thus, females may either have a higher probability of clearing an acquired oropharyngeal HPV16 infection that induced E6 antibodies or a higher likelihood of an HPV16 infection inducing E6 antibodies at another anatomical site. In fact, HPV16 E6 seroprevalences and lead times at other HPV-driven cancer sites strongly suggest the anal canal as site of infection in females conferring an elevated risk for HPV16 E6 seropositivity [40,60,66] (Brenner *et al.* in preparation). The hypothesis that an anal HPV16 infection transmitted during receptive anal intercourse might induce HPV16 E6 antibodies is further supported by the observation that males reporting same-sex intercourse in the UKB study population had an elevated risk of being E6 seropositive whereas men having sex with men apparently do not have a higher risk for HPV16-OPC [69, 76]. However, females reporting same-sex

intercourse also had a similarly elevated risk for HPV16 E6 seropositivity potentially going in line with the hypothesized more efficient genito-oral transmission from the female genitals to the oropharynx.

HPV16 and 18 L1 seroprevalence decreased with increasing age while no age trend could be observed for antibodies to the HPV16 oncoproteins. Decreasing exposure to STIs with increasing age and immunosenescence likely explain the decrease in L1 seroprevalence. Antibodies against HPV16 E6 are strongly and stably detected more than 10 years before HPV16-OPC diagnosis [15,24,27]. Thus, these proteins must be expressed by HPV16 infected cells and detected by the immune system very early during OPC tumorigenesis many years before diagnosis. However, as no precursor lesion for HPV16-OPC has been described yet, this conclusion has not been investigated mechanistically so far. The absence of a decreasing trend with age suggests that in HPV16 E6 seropositives in the general population, antibodies might also be very stable.

To further investigate the meaning of HPV16 E6 antibodies, their levels and associations with sexual behaviour in the general population, and what characterises HPV16 infections inducing HPV16 E6 antibodies, seropositive individuals should be deeply phenotyped and closely monitored over time with respect to antibody levels and should undergo regular (non-invasive) clinical work-up of anatomical sites known to be susceptible for the development of an HPV16-driven malignancy. Thereby, also the sexual behaviour(s) conferring an elevated risk for HPV16 E6 seropositivity and thus the probable route(s) of HPV16 transmission, and site(s) of HPV16 infection more likely to induce HPV16 E6 antibodies could be further investigated.

The biggest limitation of the reported study is its statistical power: 80 ($E_{6_{dis}}$) and 146 ($E_{6_{pop}}$) individuals seropositive for HPV16 E6 were included in the risk factor analysis. Thus, more detailed analyses such as associations with the number of lifetime sex partners of the same gender or ethnicity were not possible. Nevertheless, this is still the largest single study of its kind with approximately five times more individuals compared to the only other existing similar study composed of controls originating from multiple (nested) case-control studies [31].

The results obtained in this study add a new level of knowledge to the discussion on the feasibility of an HPV16 E6 serology-based HPV-OPC screening. HPV16 E6 antibody measurements in the general population, representing the potential screening population, seem to be rare markers of infection by HPV16 after increased exposure due to risky sexual behaviour or exposure by specific sexual practices. Only a minority of the HPV16 E6 seropositive individuals (9.6%, “credible” interval: 4.1%–18.0%) is expected to develop an HPV16-driven cancer (Supplementary Material 3) [21,24]. Of this minority, most individuals (70%) are expected to develop HPV16-OPC (Supplementary Material 3) [15,24,60]. Thus, further tools for risk stratification are needed to identify the individuals among HPV16 E6 seropositives at highest risk of developing HPV16-OPC. In previous seroepidemiological studies including diagnosed HPV16-OPC cases, single seropositivity for HPV16 E6 was a rare event. Most HPV16-OPC (>90%) cases were seropositive for at least one other HPV16 early antigen (mostly E2, sometimes E1, or E7) at time of diagnosis and even years before [24,28,30] while double-seropositivity to HPV16 E6 and E7 was rare in our study ($\leq 12.5\%$, Supplementary Table 16). Thus, the inclusion of antibody measurements against additional HPV16 early antigens holds potential for further risk stratification to identify individuals with a high risk to develop HPV16-driven cancer among HPV16 E6 seropositives. Additionally, enriching the screening population for HPV16 E6 seropositives using both risk factors for HPV16 E6 seropositivity and HPV-OPC (e.g. male gender, age above 45 years and risky sexual behaviour) might enhance the feasibility of an HPV16 E6 serology-based HPV-OPC screening in the future.

Author's contributions

NB Data Analysis, Investigation, Methodology, Visualization, Writing – Original Draft Preparation

AJM Conceptualization, Data Curation, Writing – Review & Editing

MH Conceptualization, Writing – Review & Editing

RA Conceptualization, Writing – Review & Editing

NA Conceptualization, Writing – Review & Editing

MP Conceptualization, Supervision, Methodology, Writing – Review & Editing

TW Conceptualization, Supervision, Methodology, Writing – Review & Editing

All authors approved the submission of the manuscript.

Declaration of Competing Interest

Dr. Brenner, Dr. Mentzer, Dr. Hill, Dr. Almond, Dr. Allen and Dr. Pawlita have nothing to disclose. Dr. Waterboer reports personal fees from MSD (Merck) Sharp & Dohme, outside the submitted work.

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Supplementary materials

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