HPV driven squamous cell head and neck cancer of unknown primary is likely to be HPV driven squamous cell oropharyngeal cancer

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Word count – 2,891 words

Conflict of interest – none

Abstract

Objectives – To compare risk factors and survival in people with oropharyngeal cancer (OPC) and cancer unknown primary (CUP).

Materials and methods – We recruited 5,511 people with head and neck cancer between 2011 and 2014. We collected data on age, gender, smoking, sexual behaviour, treatment intent, stage, co-morbidity, p16 protein overexpression and biological samples. We assessed human papillomavirus (HPV) status using serological response and p16 immunohistochemistry. We followed up participants to identify those who had died. We used Cox proportional hazards regression models to estimate survival and adjust for confounders.

Results – Of the 4,843 people with squamous cell cancer 196 had CUP – a prevalence of 4.0% (95% CI 3.5% to 4.6%). Of those people with OPC and CUP 69% (1150/1668) and 60% (106/178) respectively had HPV driven tumours. People with HPV driven tumours were likely to be younger, male, non-smokers, with higher stage disease, a history of oral sex and less co-morbidity. People with HPV negative CUP and HPV driven CUP had the survival of people with a stage II/III HPV negative OPC and a stage I/II HPV driven OPC respectively. The adjusted hazard ratio for HPV driven OPC and CUP compared with HPV negative OPC and CUP was 0.46 (95% CI 0.35 to 0.59) and 0.34 (95% CI 0.14 to 0.82) respectively.

Conclusion – HPV driven CUP is likely to be HPV driven OPC. Identifying effective methods of detecting occult OPC could improve CUP management and allow the detection of early lesions in high risk groups.

Keywords – Cancer unknown primary, oropharyngeal cancer, human papillomavirus, head and neck 5000, cohort studies, survival

Introduction

In 2012 the estimated number of new cases of oropharyngeal cancer (OPC) worldwide was 100,500 with an age standardised incidence rate of 1.4 per 100,000 for both sexes combined.[1] The incidence of OPC increased between 1983 and 2002 particularly in developed countries and at younger ages[2] and more recent data from the United States[3] and the United Kingdom suggests the incidence continues to increase.[4] Smoking and alcohol use are established major risk factors for OPC with a high attributable risk.[5]

In the last twenty years human papillomavirus (HPV) has been recognised as an important cause of OPC. [6] A high and increasing proportion of OPC in North America and Europe are HPV driven. [7] These HPV driven tumours have a better prognosis [8] and a strong serological response that reflects tumour HPV status. [9]

A small proportion (<5%) of cancers in the head and neck present as a neck lump with no primary tumour apparent at initial presentation and are referred to as carcinoma of unknown primary (CUP). [10-12] A substantial proportion of people with CUP have HPV driven tumours [13,14] with an associated strong serological response [15] and better prognosis. [13-17] Furthermore, intensive investigation shows that CUP is often occult OPC. [18,19] Some CUP arise in the nasopharynx and may be Epstein Barr virus (EBV) driven. Recent guidance suggests that CUP should be divided into three categories for staging: HPV driven CUP, EBV driven CUP and HPV negative EBV negative CUP. [20]

No previous studies have prospectively recruited people with CUP and OPC from the same population and collected data to allow comparison of risk factors and survival based on HPV status. We report findings from head and neck 5000 a large clinical cohort with detailed sociodemographic and survival data.

Patients and methods

Study population

Head and Neck 5000 is a large UK-based prospective clinical cohort study that has been described in detail previously. [21, 22] Briefly, 5,511 people with a new diagnosis of head and neck cancer were enrolled between April 2011 and December 2014. Data were collected using self-reported questionnaires and by abstracting information from the clinical notes at recruitment and 4 and 12 months later. A blood sample was collected at baseline and a formalin fixed tissue block along with a histopathology report were requested. Ethical approval for this study was given by the South West – Frenchay Regional Ethics Committee (ref: 10/H0107/57).

Case definition

We included two clinical groups - people with primary squamous cell carcinoma of the oropharynx (OPC) and people with metastatic squamous cell carcinoma to a neck node who were clinically assessed as having a cancer of unknown primary (CUP). Research staff at study centres completed a data abstraction form that recorded the clinical diagnosis. We used the following ICD codes to define the OPC (C01, C02.4, C05.1, C05.2, C05.8, C05.9, C09, C10) and CUP (C80.0) groups. Where the histopathology report was available, we cross checked the pathology report to confirm the histological diagnosis. We did not provide centres with any criteria for defining or protocol for investigating cases of CUP. We abstracted the anatomical level of the pathological lymph node in the neck for CUP cases where this was recorded in the pathology report.

Human papillomavirus status

We assessed human papillomavirus (HPV) status using two sources of data. The first was the serological response to HPV antibodies from the blood sample taken at baseline. The second was the report of the measure of p16 status (as a surrogate marker) in tumour samples conducted by local clinical centres and abstracted from the pathology forms. While p16 alone is an imperfect measure of HPV driven tumours this is what was used in clinical practice at the time we enrolled people into Head and Neck 5000. We measured HPV antibodies using a glutathione S-transferase multiplex assay carried out at the German Cancer Research Center (DKFZ) in Heidelberg, Germany. [23] We defined seropositivity as HPV16 E6 antibodies with a cut-off value of $\geq$ 1000 Median Fluorescence Intensity units (MFI). While this definition is sensitive and specific and widely used it may not capture all tumours driven by HPV16 and by HPV other serotypes. So we have included a sensitivity analysis using an extended approach that we have described previously [15] to define seropositivity using additional criteria for HPV16 and for HPV18, HPV31, HPV33, HPV35, HPV45, HPV52 and HPV58. We have provided details of these criteria in Supplementary Table 1.

Validation of the HPV status in people with CUP

In a subset of participants with CUP where a formalin fixed paraffin embedded (FFPE) tissue sample had been supplied by the study centres we extracted DNA and RNA. [24] We prepared and reviewed hematoxylin and eosin stained slides before and after sectioning to ensure that tumour was present in sectioned tissue. We included negative controls to monitor cross-contamination. HPV DNA analysis was conducted

using Multiplex Papillomavirus Genotyping. [25,26] Samples positive for HPV and/or beta-globin were considered to be DNA valid. We performed HPV RNA analysis, that is, detection of viral transcripts, by HPV type-specific reverse transcription polymerase chain reaction (RT-PCR) and hybridization assays, [24] which amplify HPV E6*I and ubiquitin C cDNA as a cellular mRNA quality control. We considered specimens that were HPV E6*I and/or ubiquitin C mRNA-positive to be RNA valid. Our gold standard for an HPV driven tumour was one that was both DNA and RNA positive.

Measurement of Epstein Barr virus status in people with CUP

We assessed Epstein Barr virus (EBV) status through qualitative identification of latent EBV infection using in-situ hybridisation (ISH) carried out on formalin fixed paraffin embedded tissue sections (3µm). We used a clinically validated automated BOND system. [27] When a tissue block was not available we abstracted information from the pathology reports provided by the diagnostic centre.

Socio-demographic and lifestyle measures

We used data from baseline health and lifestyle questionnaires to describe socio-demographic and lifestyle measures of participants including age, gender, smoking and sexual behaviour. We calculated age using date of study consent and date of birth. We categorised smoking status as never, current or former smokers (previously smoked at least 100 cigarettes up until one year before diagnosis). We used responses to the question "*how many different sexual partners have you performed oral sex on?*" to group participants into three categories: never performed oral sex; one to five oral sex partners and six or more oral sex partners.

Clinical measures

Research staff in local clinical centres abstracted information from medical notes. We derived the clinical staging of the tumour from T (characteristics of the tumour site), N (degree of lymph node involvement) and M (the absence or presence of metastases) based on the American Head and Neck Society TNM staging of head and neck cancer to compare stage between HPV seropositive and seronegative tumours. [28] We also used the staging proposed by the International Collaboration on Oropharyngeal Cancer Network for staging analyses of HPV seropositive tumours. [29] We used the Adult Comorbidity Evaluation 27 (ACE 27) [30] form completed by research staff in clinical centres to record the presence and severity of medical comorbidities. We grouped participants into four categories: no co-morbidity, mild comorbidity; moderate decompensation and severe decompensation. We split participants into two groups based on treatment intent: those treated with curative intent, or those treated with palliative or supportive intent. We used treatment information collected at four months after recruitment. There were seven categories: 1. surgery alone; 2. chemotherapy alone; 3. radiotherapy alone; 4. surgery and radiotherapy; 5. surgery and chemoradiotherapy; 6. chemotherapy +/- surgery and 7. no treatment.

Mortality follow-up

We flagged study participants with the UK Health and Social Care Information Centre and they provided regular notification of the date and cause of death of participants who had died. We measured survival time from study enrolment until either death or the end of the most recent follow-up period.

Statistical analysis

We calculated the sensitivity, specificity and positive predictive value for HPV serology and clinical p16 measures in people with CUP. We carried out analyses for OPC and CUP separately. We calculated 95% confidence intervals for proportions. We used chi-squared tests and student's T tests to assess statistical evidence of difference between categorical variables and continuous variables respectively in HPV seropositive and seronegative groups. We plotted unadjusted Kaplan-Meier graphs to compare overall survival between HPV seronegative and HPV seropositive cancers with survival for OPC stratified by cancer stage. We used Cox proportional hazards regression models to estimate survival and to adjust for age, gender, stage (except CUP), treatment intent, co-morbidity and smoking. We repeated the unadjusted analyses for those with complete data for confounders used in the adjusted model. We performed a sensitivity analysis using an extended definition of seropositivity. We used the Head and Neck 5000 dataset version 2.5 in this study. All statistical analyses were performed using Stata 15.0. [31]

Results

Number recruited to the study

We have summarised the number of subjects in the study in Figure 1. We had data on HPV status on 178 (91%) of the 196 CUP cases and on 1668 (88%) of the 1885 OPC cases.

Prevalence of CUP and clinical characteristics

The prevalence of CUP SCC was 196/4,843 = 4.0% (95% CI 3.5% to 4.6%). In the 103 people with CUP where we had data on the side and neck level 54 nodes were on the left and 49 were on the right. Ninety (87%) were located in level II, five in level I, six in level III and two in level IV suggesting that most were likely to have arisen from cancers in the head and neck.

Validation of HPV status in people with CUP

As the ability of serology and p16 to identify HPV driven OPC is established we defined an OPC as HPV driven if it was seropositive or if serum was not available if the clinical p16 was positive. The use of clinical p16 though imperfect allowed us to identify tumours that were likely to be HPV driven or not where serological data were not available to reduce the numbers with missing data and so increase the power of our analyses. To confirm the utility of this definition in CUP we compared the results of serology, tissue measures of HPV DNA and RNA and p16 immunohistochemistry in Figure 2. We had formalin fixed tissue available on 49 people with CUP. In 37 of these 49 people both DNA and RNA were positive. No-one with a tissue HPV negative tumour was seropositive but three people with tissue HPV positive tumours

were seronegative. The sensitivity, specificity and positive predictive value for serology based on HPV16 E6 >1000 were 91%, 100% and 100% respectively. Two of these three were considered seropositive when we used the extended definition of seropositivity. Both of these cases were HPV33 positive. All tissue HPV positive tumours were p16 positive but two tissue HPV negative tumours were p16 positive. The sensitivity, specificity and positive predictive value for clinical p16 were 100%, 71% and 93% respectively. Based on these assay characteristics we used the same definition for HPV driven tumours in OPC and CUP.

EBV status in people with CUP

We had research EBER-ISH data available from formalin fixed tissue on 51 people with CUP and clinical EBER-ISH available from histopathological reports on 18 people with CUP – a total of 64 people with CUP. Only one person with CUP was positive for EBER-ISH. The EBER-ISH positive case had a level 2 lymph node as was thought to have nasopharyngeal cancer but no primary lesion could be identified.

Characteristics of people with OPC and CUP

We present a comparison of baseline characteristics of people with OPC and CUP stratified by HPV status in Table 1. For people with OPC the prevalence of HPV positive tumours was 69% (1150/1668) and for people with CUP the prevalence of HPV positive tumours was 60% (106/178). People with HPV positive OPC were more likely than those with HPV negative OPC to be younger, male, higher stage, treated with curative intent, treated with chemoradiotherapy, to have a past history of more oral sex partners and not smoking and were less likely to have co-morbidity.

People with HPV positive CUP showed similar differences compared to those with HPV negative CUP.

Survival comparisons between HPV positive and negative OPC and CUP

We observed 448 deaths amongst the 1,668 people with OPC after a mean follow-up time of 4.2 years and 48 deaths amongst the 178 people with CUP after a mean follow-up time of 4.1 years. We described survival for HPV positive and negative OPC and CUP in Figure 3. We then compared survival for HPV negative and positive OPC and CUP stratified by stage in Figures 4 and 5 respectively. As only three participants with the HPV positive OPC were ICONS stage IV this group was combined with stage III for the analysis. Figure 3 shows that survival for HPV negative OPC and CUP is similar and that survival for HPV positive OPC and CUP is also similar. Figure 4 shows that HPV negative CUP has a similar survival to a stage II or III HPV negative OPC. Figure 5 shows that HPV positive CUP has a similar survival to a stage I or II HPV positive OPC. We present the difference in survival for OPC and CUP by HPV status in Table 2. The survival benefit associated with being HPV positive is similar in both groups in unadjusted analyses. There is modest attenuation on adjustment for a range of potential confounding variables and reduction in the number of cases included in the analysis leading to wider confidence intervals.

Sensitivity analysis

We repeated the analyses using our extended HPV serology definition – the prevalence of HPV positivity was increased by 3% in both OPC and CUP. For people with OPC the prevalence of HPV positive tumours was 72% (1208/1668) and for people with CUP the prevalence of HPV positive tumours was 63% (112/178). The other results were all similar (see supplementary Tables 2 and 3).

Discussion

In this large prospective clinical cohort study the prevalence of CUP among all squamous cell cancers of the head and neck reported by study centres at baseline was around 4%. Of these, 60% of CUP were HPV driven. Based on similarities in risk factor profile and survival our data suggest that people with HPV driven CUP have HPV driven OPC. While people with HPV negative CUP are likely to have HPV negative OPC they could also have a primary elsewhere in the head and neck.

The prevalence of CUP in our study was similar to that reported in other studies [10-12] as was the prevalence of CUP that were HPV driven. [14] Our observation that serology can identify HPV driven CUP confirms a previous report [15] and is unsurprising as they are likely to represent HPV driven OPC. Our observation that people with HPV driven CUP have improved survival is consistent with that reported in a recent meta-analysis. [14] Our study was the first prospective study to recruit CUP and OPC cases contemporaneously. One previous retrospective study matched people with CUP to people with early stage OPC and reported similar survival between the two groups.[32]

Our results support guidance that HPV status should be checked in people with CUP. [33] HPV positive CUP should be treated as HPV driven OPC after investigations have excluded other sites in the head and neck and sought to identify the site of the primary lesion in the oropharynx. [33] Further our findings support the conclusion by Ross et al [32] that HPV driven CUP could potentially be included in trials of HPV driven OPC.

People with CUP may offer an ideal group to develop and test more effective techniques to identify occult OPC (as investigation is justified because it may alter treatment). Identification of early stage disease is a necessary first step in the development of screening programmes based on seroconversion.

Our study had several strengths. First, this was a large prospective population-based observational study that reflects practice. Second, we recruited people with CUP and OPC contemporaneously. Third, we collected detailed data on health and lifestyle, HPV status and outcome. Our study also had a number of weaknesses. First, although the study was large the number of people with CUP enrolled was modest. Second, the diagnosis and investigation of CUP was not standardised. Third, those who participated and who completed questionnaires may not have been representative of all those eligible. Finally, clinical data on HPV status, EBV status and extra nodal extension required for staging were not recorded on many histopathology forms and p16 alone was used to identify HPV driven tumours in clinical practice. We were able to assess HPV status using serology for most people. Furthermore, based on those tested we conclude that few people with CUP had EBV driven cancers. Data were collected before staging of CUP was recommended so we were not able to confidently stage cases of CUP.

Conclusion

We showed that HPV driven CUP is likely to be HPV driven OPC and that HPV negative CUP may well be HPV negative OPC. Efforts to find early OPC lesions (and early lesions at other sites) are justified to optimise care and improve prognosis. Further research to develop and test more effective methods of detecting occult OPC could improve management of people with CUP and allow the detection of early lesions in high risk groups.

Acknowledgements

We would like to thank the people with head and neck cancer who took part in this study. We would also like to thank the research, laboratory and clinical staff who supported this study. In particular, we would like to thank Elisa Cappio Barazzone, Marianna Elena Estrada Estrada, Teresa Marker, Lisa-Marie Schneider and Evi Schyr for excellent technical support. This publication presents data from the Head and Neck 5000 study. The study was a component of independent research funded by the National Institute for Health Research (NIHR) under its Programme Grants for Applied Research scheme (RP-PG-0707-10034). The views expressed in this publication are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health. Core funding was also provided through awards from Above and Beyond, University Hospitals Bristol Research Capability Funding and the NIHR Senior Investigator award to Professor Andy Ness. Human papillomavirus (HPV) serology was supported by a Cancer Research UK Programme Grant, the Integrative Cancer Epidemiology Programme (grant number: C18281/A19169).

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Figure legends

Figure 1 **Summary of people with oropharyngeal cancer and cancer of unknown primary with serology or clinical P16 data**

Figure 2 **- Heat map displaying agreement between HPV DNA alone, HPV RNA alone, HPV serology using HPV16 E6>1000, an extended definition of HPV serology and p16**

Figure 3 **- Kaplan Meier plots for OPC and CUP stratified by HPV status**

Figure 4 **- Kaplan Meier plots for HPV negative OPC stratified by stage and HPV negative CUP**

Figure 5 **- Kaplan Meier plots for HPV positive OPC stratified by ICONS stage and HPV positive CUP**

Table 1 **- Risk factor profiles for HPV driven and HPV negative SCC of the oropharynx and cancer of unknown primary**

	Oropharynx			Primary of unknown origin		
	Negative	**Positive**	**p**	**Negative**	**Positive**	**p**
N	518	1,150	-	72	106	-
Age, mean (SD)	61.0 (9.5)	58.0 (8.7)	<0.001	63.4 (10.8)	55.6 (8.4)	<0.001
Age group			<0.001			
Less than 45	19 (3.7%)	60 (5.2%)		2 (2.8%)	8 (7.5%)	
45 to 54	105 (20.3%)	334 (29.0%)		14 (19.4%)	41 (38.7%)	
55 to 64	205 (39.6%)	496 (43.1%)		22 (30.6%)	39 (36.8%)	
65 to 74	153 (29.5%)	210 (18.3%)		20 (27.8%)	17 (16.0%)	
75 and over	36 (6.9%)	50 (4.3%)		14 (19.4%)	1 (0.9%)	
Sex			0.022			0.150
Male	388 (74.9%)	919 (79.9%)		55 (76.4%)	90 (84.9%)	
Female	130 (25.1%)	231 (20.1%)		17 (23.6%)	16 (15.1%)	
TNM staging			<0.001			-
I	48 (9.3%)	20 (1.7%)		-	-	
II	82 (15.9%)	86 (7.5%)		-	-	
III	78 (15.1%)	153 (13.4%)		-	-	
IV	308 (59.7%)	886 (77.4%)		-	-	
ICON-S staging			-			-
I	-	653 (58.5%)		-	-	
II	-	235 (21.1%)		-	-	
III	-	225 (19.7%)		-	-	
IV	-	3 (0.3%)		-	-	
Treatment intent			<0.001			0.042
Curative	493 (95.2%)	1,146 (99.7%)		66 (91.7%)	104 (98.1%)	
Palliative/supportive	25 (4.8%)	4 (0.3%)		6 (8.3%)	2 (1.9%)	
Treatment group			<0.001			0.190
Surgery only	47 (9.1%)	30 (2.6%)		7 (9.7%)	5 (4.7%)	
Chemoradiotherapy only	253 (48.8%)	659 (57.3%)		25 (34.7%)	50 (47.2%)	
Radiotherapy only	107 (20.7%)	106 (9.2%)		13 (18.1%)	12 (11.3%)	
Surgery and radiotherapy	48 (9.3%)	127 (11.0%)		14 (19.4%)	12 (11.3%)	
Surgery and chemoradiotherapy	51 (9.8%)	225 (19.6%)		11 (15.3%)	23 (21.7%)	
Chemotherapy +/- surgery	9 (1.7%)	1 (0.1%)		1 (1.4%)	4 (3.7%)	
No treatment	3 (0.6%)	2 (0.2%)		1 (1.4%)	0 (0.0%)	
Comorbidity index			<0.001			0.021
No comorbidity	172 (33.8%)	599 (53.1%)		31 (43.7%)	65 (64.4%)	
Mild	194 (38.1%)	377 (33.4%)		20 (28.2%)	24 (23.8%)	
Moderate	115 (22.6%)	133 (11.8%)		15 (21.1%)	10 (9.9%)	
Severe	28 (5.5%)	20 (1.8%)		5 (7.0%)	2 (2.0%)	
Performed oral sex			<0.001			<0.001
Never performed	42 (14.9%)	34 (5.0%)		11 (26.8%)	4 (5.6%)	
1-5 people	176 (62.4%)	382 (55.8%)		23 (56.1%)	35 (48.6%)	
6+ people	64 (22.7%)	269 (39.3%)		7 (17.1%)	33 (45.8%)	
Tobacco			<0.001			0.110
Current user	142 (40.6%)	66 (7.7%)		10 (22.2%)	9 (10.1%)	
Former	166 (47.4%)	496 (58.2%)		25 (55.6%)	50 (56.2%)	
Never	42 (12.0%)	290 (34.0%)		10 (22.5%)	30 (33.7%)	

Table 2 - Survival by HPV status in people with oropharyngeal cancer (OPC) and cancer of unknown primary (CUP)

	Oropharynx (OPC)		Cancer of unknown primary (CUP)	
	HR	p-value	HR	p-value
All cases – unadjusted				
HPV status	n = 1,668	<0.001	n = 178	<0.001
Negative	1.00 (Ref.)		1.00 (Ref.)	
Positive	0.33 (0.27, 0.39)		0.33 (0.19, 0.60)	
Complete cases – unadjusted [a]				
HPV status	n = 1,174	<0.001	n = 128	0.001
Negative	1.00 (Ref.)		1.00 (Ref.)	
Positive	0.35 (0.28, 0.44)		0.29 (0.14, 0.62)	
Complete cases – adjusted [b]				
HPV status	n = 1,174	<0.001	n = 128	0.017
Negative	1.00 (Ref.)		1.00 (Ref.)	
Positive	0.46 (0.35, 0.59)		0.34 (0.14, 0.82)	

[a] OPC complete cases defined as having complete data for age, sex, stage, treatment modality, co-morbidity and smoking. *CUP complete cases defined as having complete data for age, sex, treatment modality, co-morbidity and smoking.
[b] OPC models adjusted by age, sex, stage, treatment modality, co-morbidity and smoking. CUP models adjusted by age, sex, treatment modality, co-morbidity and smoking.

Figure 1

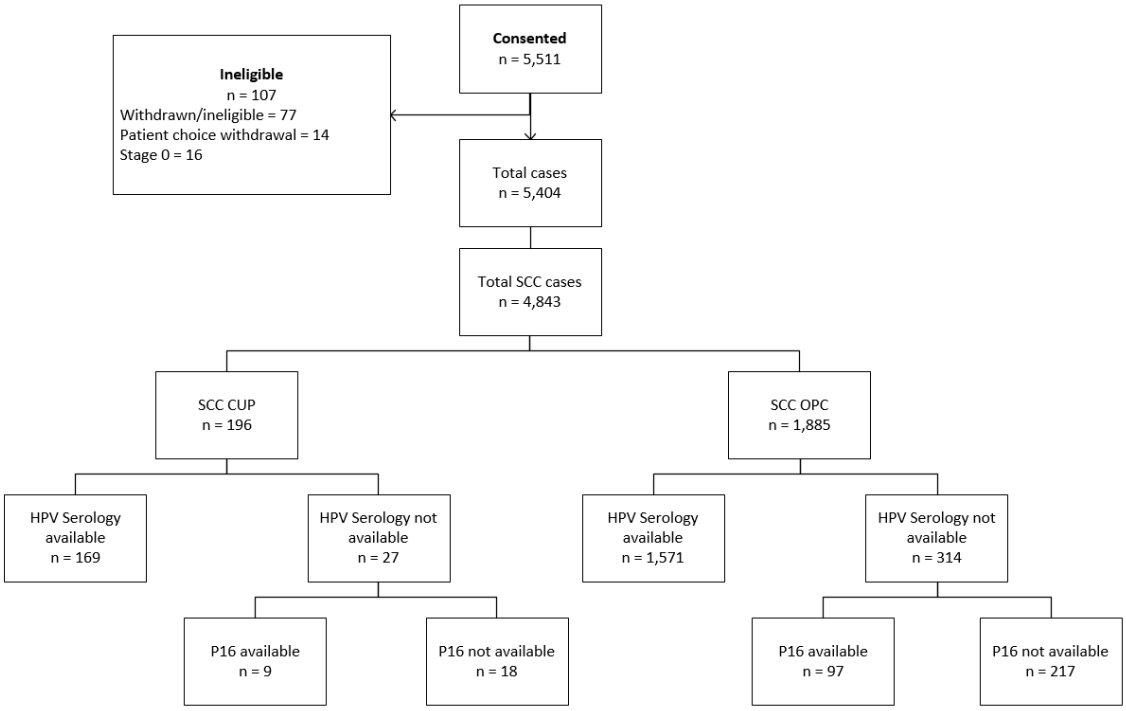
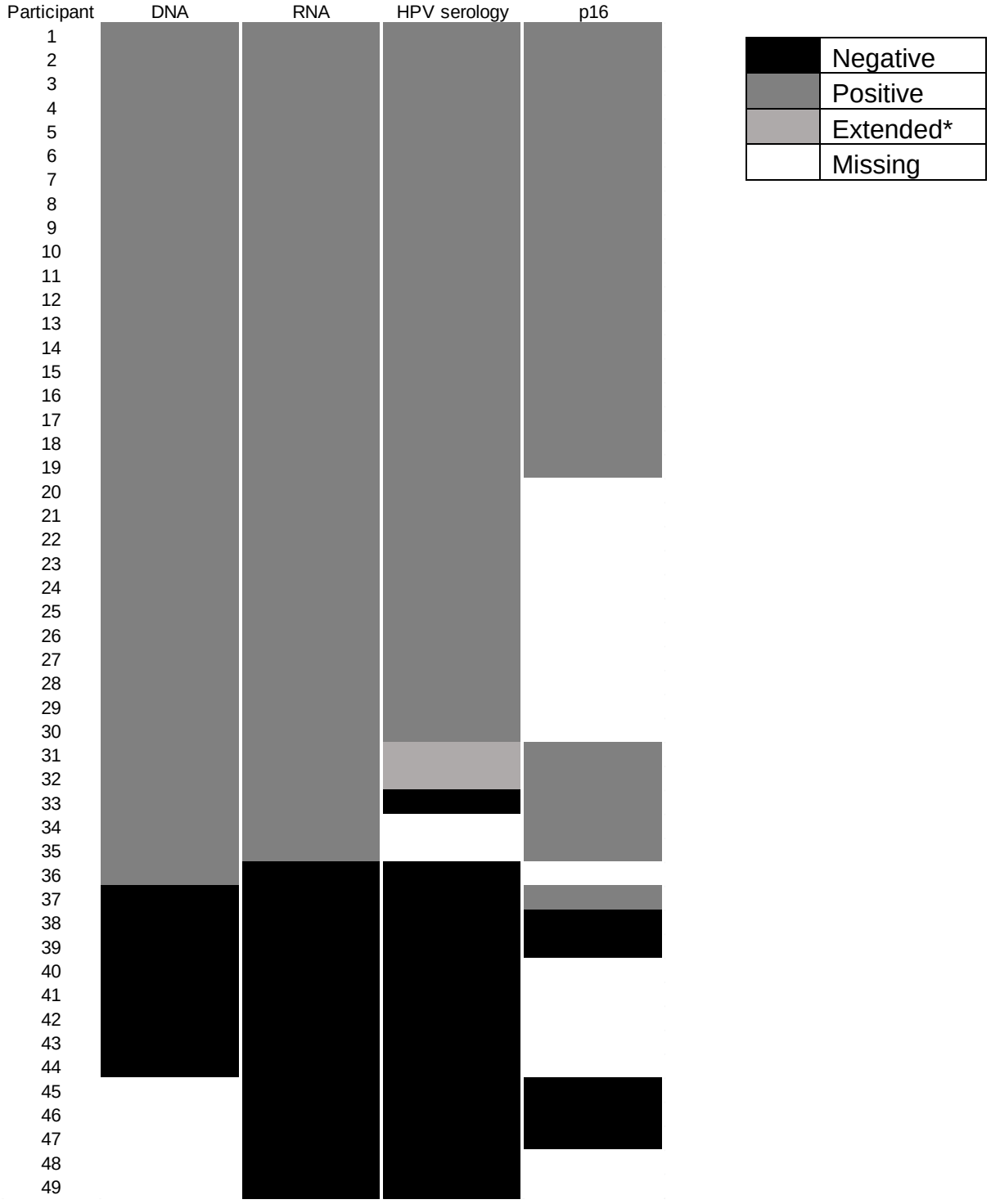

Consented
n = 5,511
Ineligible
n = 107
Withdrawn/ineligible = 77
Patient choice withdrawal = 14
Stage 0 = 16
Total cases
n = 5,404
Total SCC cases
n = 4,843
SCC CUP
n = 196
SCC OPC
n = 1,885
HPV Serology available
n = 169
HPV Serology not available
n = 27
HPV Serology available
n = 1,571
HPV Serology not available
n = 314
P16 available
n = 9
P16 not available
n = 18
P16 available
n = 97
P16 not available
n = 217

Figure 2



* HPV serology positive is based on HPV16 E6 >1000 MFI and the extended category includes cases that are negative using HPV16 E6 >1000 but positive using the extended HPV definition. The two additional positive cases were positive to HPV 33.

Figure 3

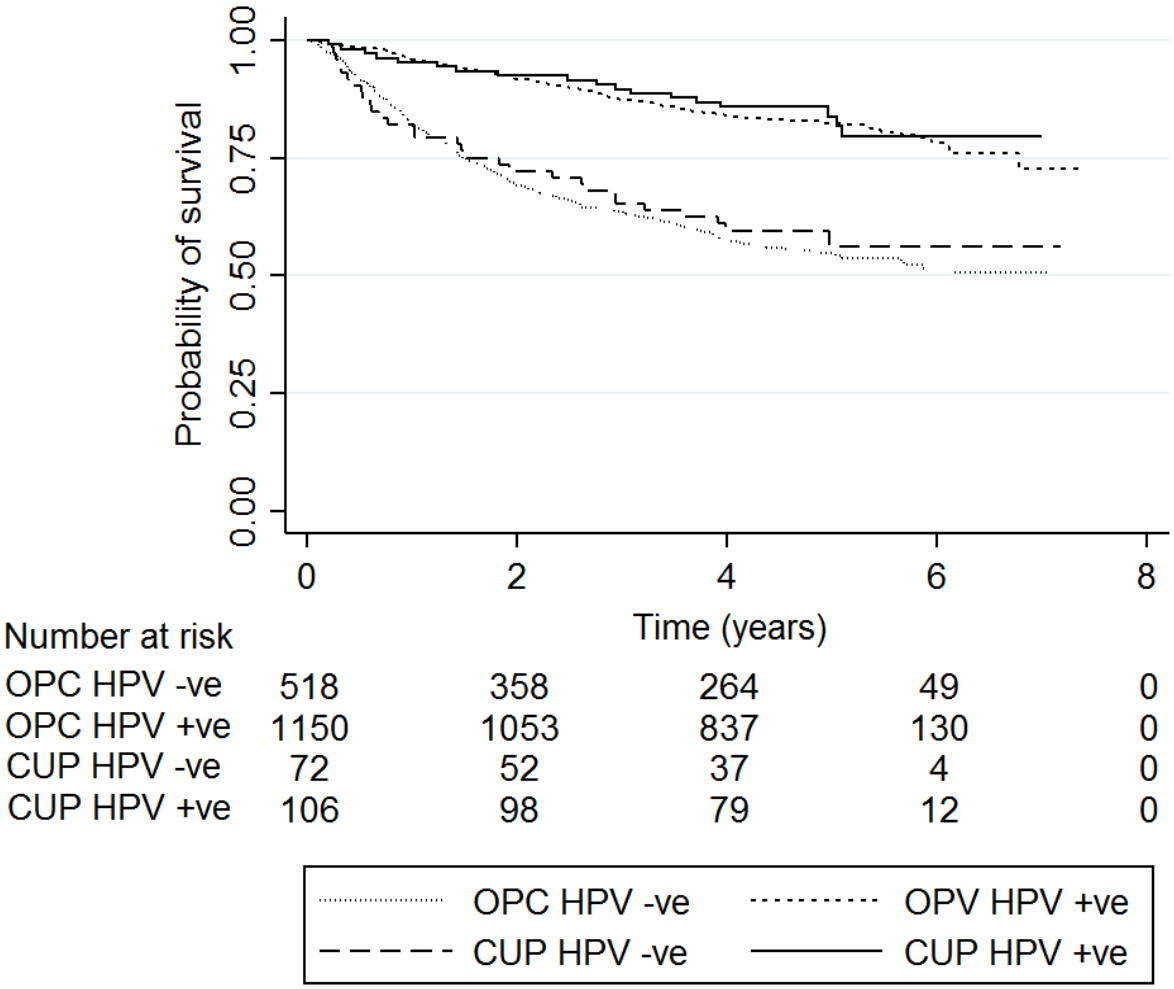
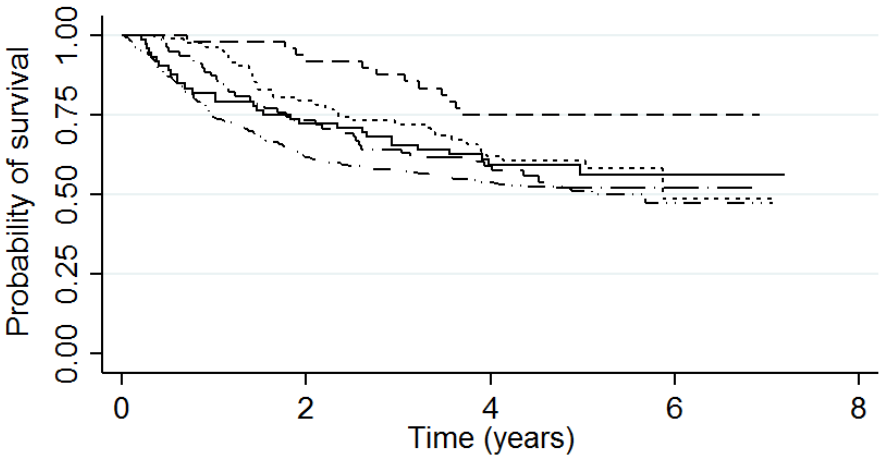

Probability of survival
0.00
0.25
0.50
0.75
1.00
0
2
4
6
8
Time (years)
Number at risk
OPC HPV -ve 518 358 264 49 0
OPC HPV +ve 1150 1053 837 130 0
CUP HPV -ve 72 52 37 4 0
CUP HPV +ve 106 98 79 12 0
OPC HPV -ve
OPV HPV +ve
CUP HPV -ve
CUP HPV +ve

Figure 4



Number at risk					
OPC HPV -ve stage I	48	44	32	6	0
OPC HPV -ve stage II	82	65	45	9	0
OPC HPV -ve stage III	78	57	42	10	0
OPC HPV -ve stage IV	308	190	145	24	0
CUP HPV -ve	72	52	37	4	0

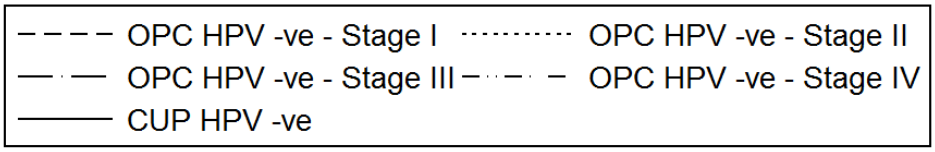
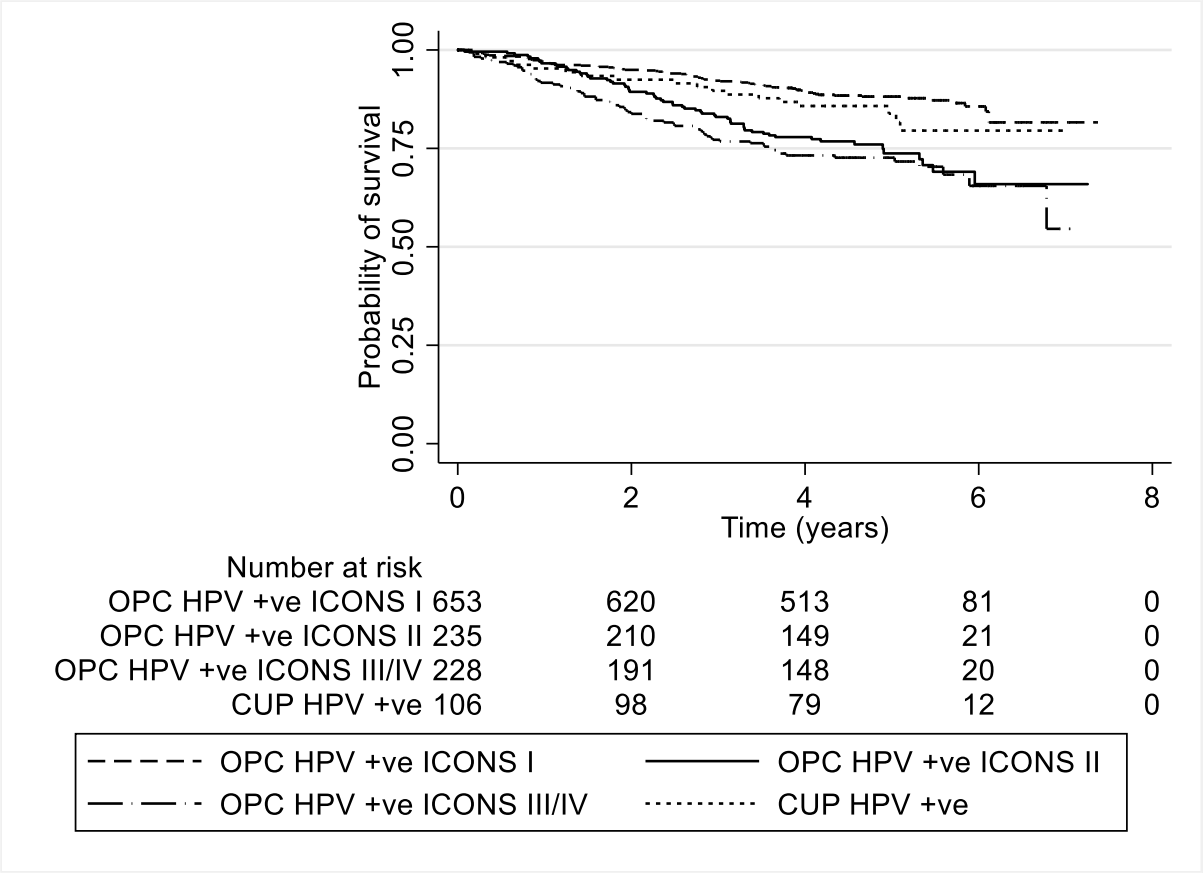


Figure 5


Probability of survival
0.00
0.25
0.50
0.75
1.00
0
2
4
6
8
Time (years)
Number at risk
OPC HPV +ve ICONS I 653 620 513 81 0
OPC HPV +ve ICONS II 235 210 149 21 0
OPC HPV +ve ICONS III/IV 228 191 148 20 0
CUP HPV +ve 106 98 79 12 0
OPC HPV +ve ICONS I
OPC HPV +ve ICONS II
OPC HPV +ve ICONS III/IV
CUP HPV +ve

Supplementary Table S1 **– Criteria for defining HPV seropositivity used in the sensitivity analysis (online only)**

Serotype	**Criteria ***
HPV16	E6 ≥ 1000
HPV16	3 of 4 (E1 >200, E2 >679, E6 >484 & <1000, E7 >548)
HPV 18	3 of 4 (E1 >200, E2 >600, E6 >243, E7 >789)
HPV 18	E6>243 and E7>789
HPV31	E6 >890 and E7 >500
HPV33	E6 >253 and E7 >500
HPV35	E6 >271 and E7 >384
HPV45	E6 >249 and E7 >200
HPV52	E6 >271 and E7 >200
HPV58	E6 >250 and E7 >200

***** The values are for antibodies measured in Median Fluorescence Intensity units (MFI)

Supplementary Table S2 **- Risk factor profiles for HPV driven SCC of the oropharynx and cancer unknown primary using HPV all and P16**

	Oropharynx			Primary of unknown origin		
	Negative	**Positive**	**p**	**Negative**	**Positive**	**p**
N	460	1,208	-	66	112	
Age, mean (SD)	61.1 (9.6)	58.2 (8.7)	<0.001	64.7 (10.1)	55.3 (8.4)	<0.001
Age group			<0.001			
Less than 45	17 (3.7%)	62 (5.1%)		0 (0.0%)	10 (8.9%)	
45 to 54	91 (19.8%)	348 (28.8%)		12 (18.2%)	43 (38.4%)	
55 to 64	182 (39.6%)	519 (43.0%)		20 (30.3%)	41 (36.6%)	
65 to 74	136 (29.6%)	227 (18.8%)		30 (30.3%)	17 (15.2%)	
75 and over	34 (7.4%)	52 (4.3%)		14 (21.2%)	1 (0.9%)	
Sex			0.014			0.480
Male	342 (74.3%)	965 (79.9%)		52 (78.8%)	93 (83.0%)	
Female	118 (25.7%)	243 (20.1%)		14 (21.2%)	19 (17.0%)	
TNM staging			<0.001			-
I	48 (10.5%)	20 (1.7%)		-	-	
II	79 (17.2%)	89 (7.4%)		-	-	
III	69 (15.1%)	162 (13.5%)		-	-	
IV	262 (57.2%)	932 (77.5%)		-	-	
ICON-S staging			-			-
I	-	653 (58.5%)		-	-	
II	-	235 (21.1%)		-	-	
III	-	225 (19.7%)		-	-	
IV	-	3 (0.3%)		-	-	
Treatment intent			<0.001			0.023
Curative	437 (95.0%)	1,102 (99.5%)		60 (90.9%)	110 (98.2%)	
Palliative/supportive	23 (5.0%)	6 (0.5%)		6 (9.1%)	2 (1.8%)	
Treatment group			<0.001			0.140
Surgery only	47 (10.2%)	30 (2.5%)		7 (10.6%)	5 (4.5%)	
Chemoradiotherapy only	216 (47.0%)	696 (57.6%)		23 (34.8%)	52 (46.4%)	
Radiotherapy only	101 (22.0%)	112 (9.3%)		12 (18.2%)	13 (11.6%)	
Surgery and radiotherapy	43 (9.3%)	132 (10.9%)		13 (19.7%)	13 (11.6%)	
Surgery and chemoradiotherapy	41 (8.9%)	235 (19.5%)		9 (13.6%)	25 (22.3%)	
Chemotherapy +/- surgery	9 (2.0%)	1 (0.1%)		1 (1.5%)	4 (3.6%)	
No treatment	3 (0.7%)	2 (0.2%)		1 (1.5%)	0 (0.0%)	
Comorbidity index			<0.001			0.003
No comorbidity	150 (33.2%)	621 (52.4%)		26 (40.0%)	70 (65.4%)	
Mild	165 (36.5%)	406 (34.2%)		19 (29.2%)	25 (23.4%)	
Moderate	112 (24.8%)	136 (11.5%)		15 (23.1%)	10 (9.3%)	
Severe	25 (5.5%)	23 (1.9%)		5 (7.7%)	2 (1.9%)	
Performed oral sex			<0.001			<0.001
Never performed	39 (15.8%)	37 (5.1%)		11 (30.6%)	4 (5.2%)	
1-5 people	158 (64.0%)	400 (55.6%)		19 (52.8%)	39 (50.6%)	
6+ people	50 (20.2%)	283 (39.3%)		6 (16.7%)	34 (44.2%)	
Tobacco			<0.001			0.037
Current user	138 (44.7%)	70 (7.8%)		10 (25.0%)	9 (9.6%)	
Former	144 (46.6%)	518 (58.0%)		22 (55.0%)	53 (56.4%)	
Never	27 (8.7%)	305 (34.2%)		8 (20.0%)	32 (34.0%)	

Supplementary Table S3 **- Survival by HPV status using HPV all and P16**

	Oropharynx		**Primary of unknown origin**	
	HR	p-value	HR	p-value
All cases – unadjusted		<0.001		<0.001
HPV status	n = 1,668		n = 178	
Negative	1.00 (Ref.)		1.00 (Ref.)	
Positive	0.28 (0.24, 0.34)		0.30 (0.17, 0.54)	
Complete cases – unadjusted [a]		<0.001		<0.001
HPV status	n = 1,174		n = 128	
Negative	1.00 (Ref.)		1.00 (Ref.)	
Positive	0.30 (0.24, 0.38)		0.23 (0.11, 0.49)	
Complete cases – adjusted [b]		<0.001		<0.001
HPV status	n = 1,174		n = 128	
Negative	1.00 (Ref.)		1.00 (Ref.)	
Positive	0.36 (0.27, 0.47)		0.28 (0.11, 0.70)	

[a] OPC complete cases defined as having complete data for age, sex, stage, treatment modality, co-morbidity and smoking.
[a] PUO complete cases defined as having complete data for age, sex, treatment modality, co-morbidity and smoking.
[b] OPC models adjusted by age, sex, stage, treatment modality, co-morbidity and smoking. PUO models adjusted by age, sex, treatment modality, co-morbidity and smoking.