

Development and validation of a combined hypoxia and immune prognostic classifier for head and neck cancer

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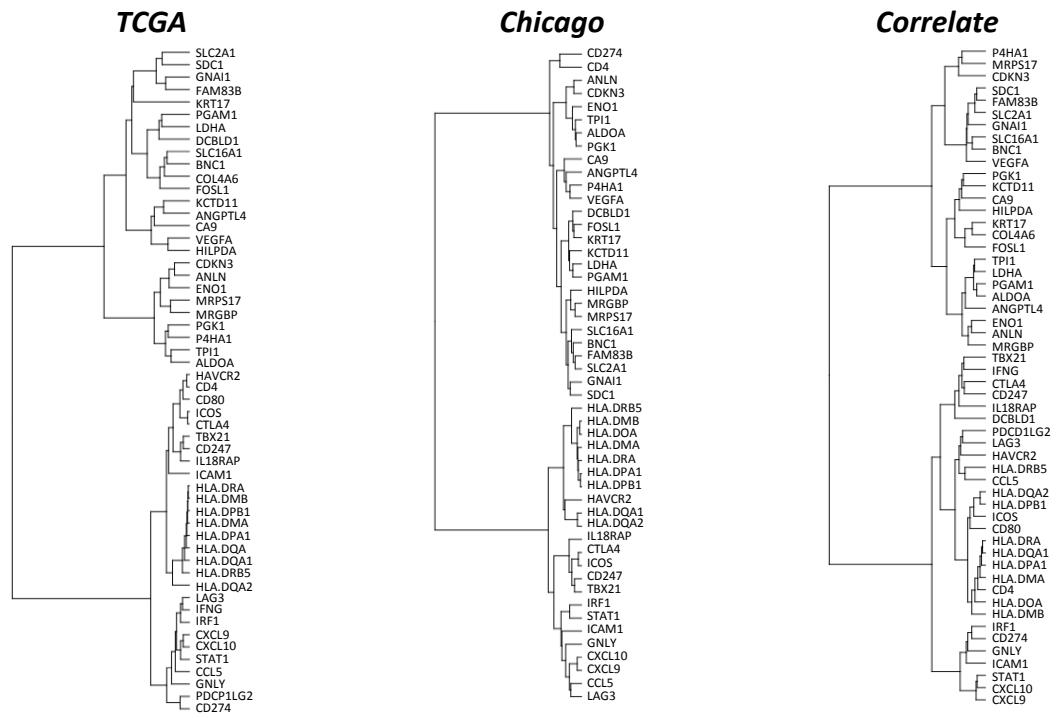
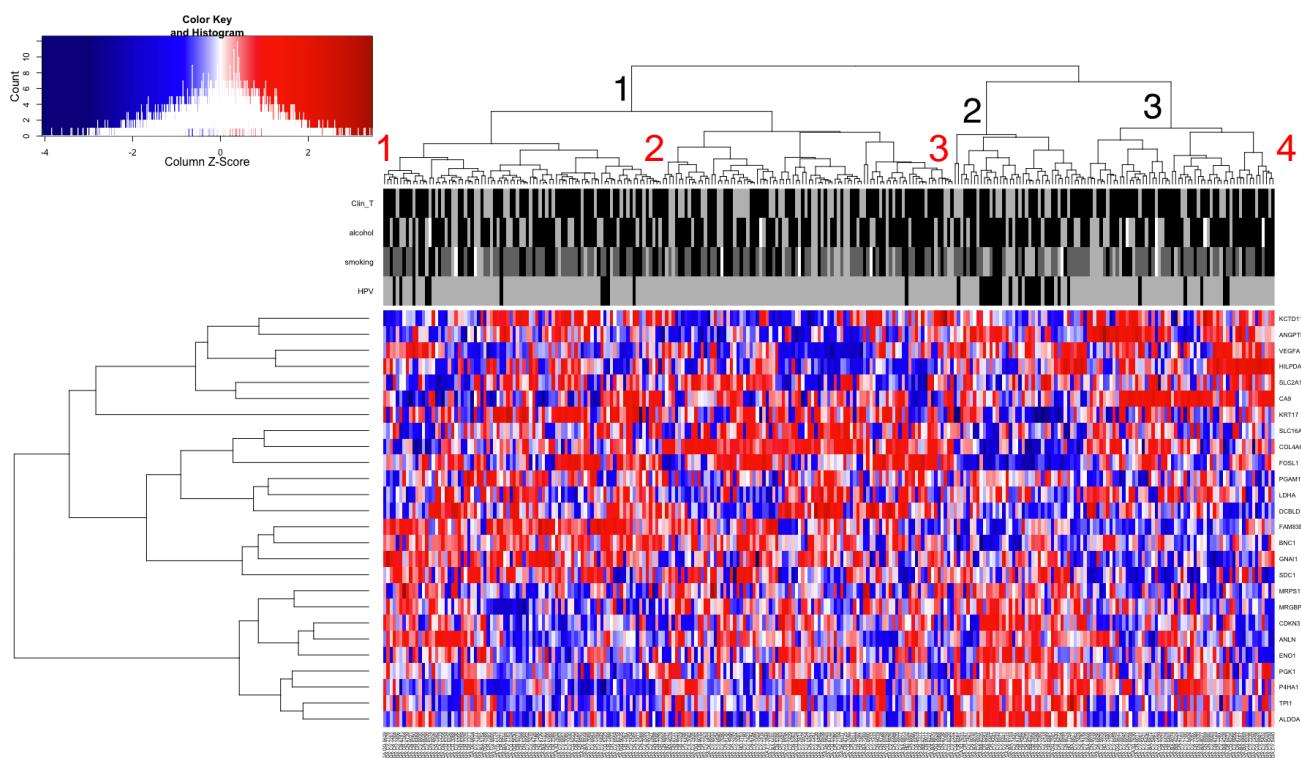
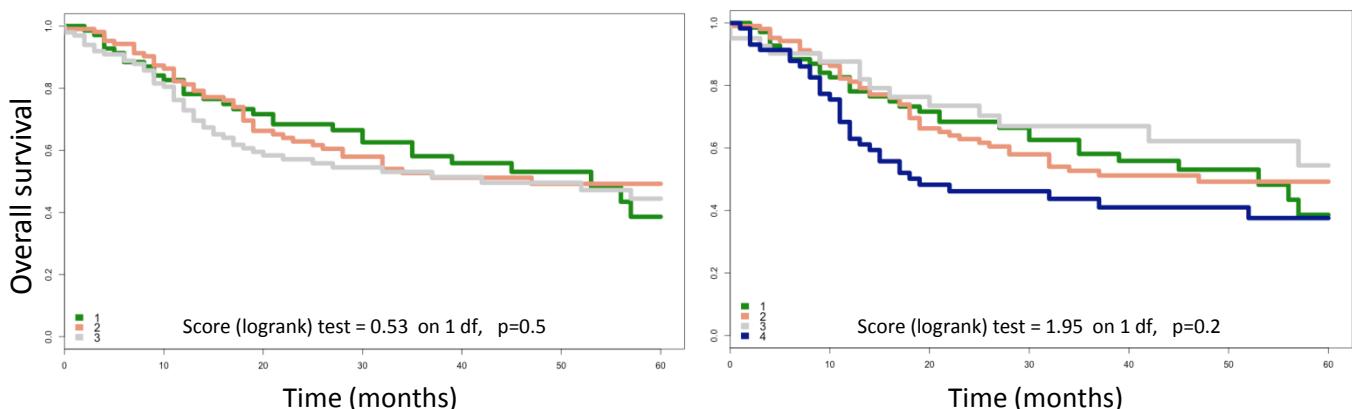


Figure S1. Gene order within the cluster defined groups.

A

Eustace hypoxia signature

**B**

C

CIRC immune signature

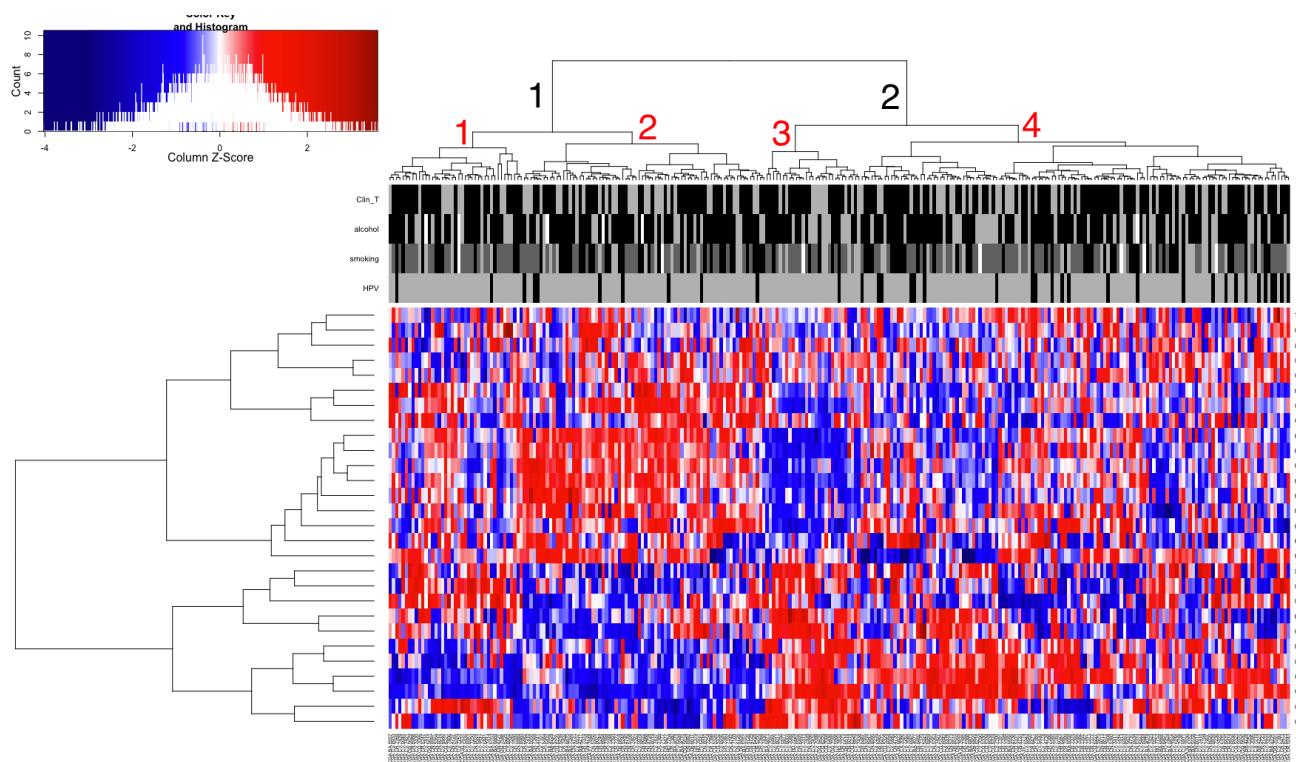
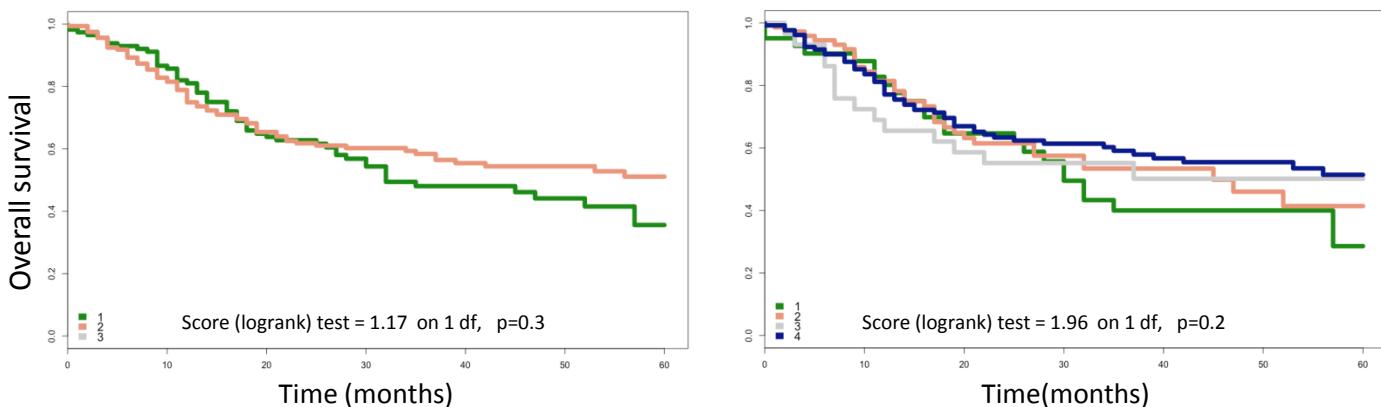
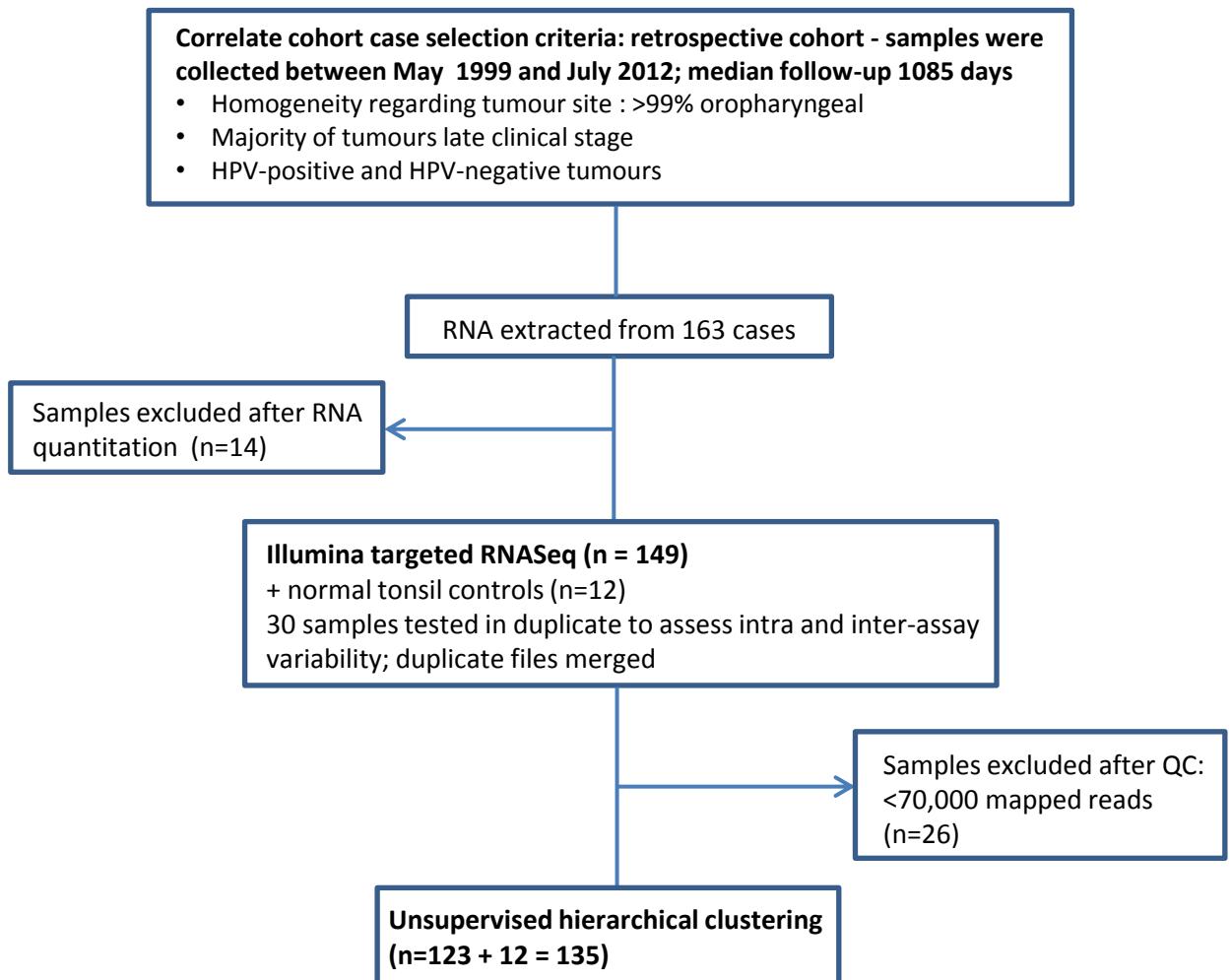
**D**

Figure S2. Analysis of individual hypoxia and immune gene signatures. Two dimensional hierarchical clustering of (A) hypoxia- and (C) immune response-related genes for the TCGA dataset does not identify comparable HNC patient subgroups with co-ordinate gene expression. (B and D) Kaplan-Meier survival plots for OS stratified according to the heatmap cluster-defined subgroups, for (B) Eustace hypoxia signature analysed using three or four highest hierarchical sample groups, and (D) CIRC immune signature analysed using two or four groups. Data are censored at 5 years.

Figure S3. Flow diagram showing progress of patient samples through study for Correlate cohort



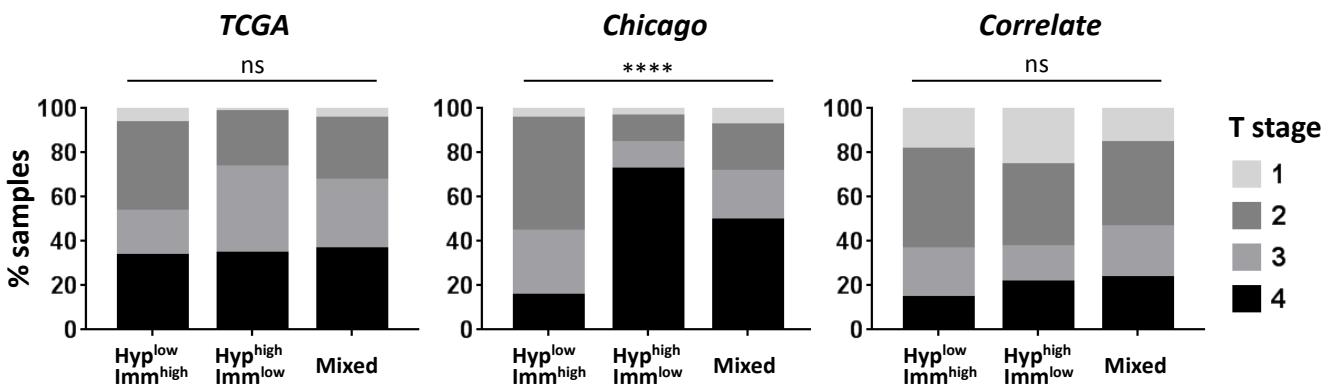
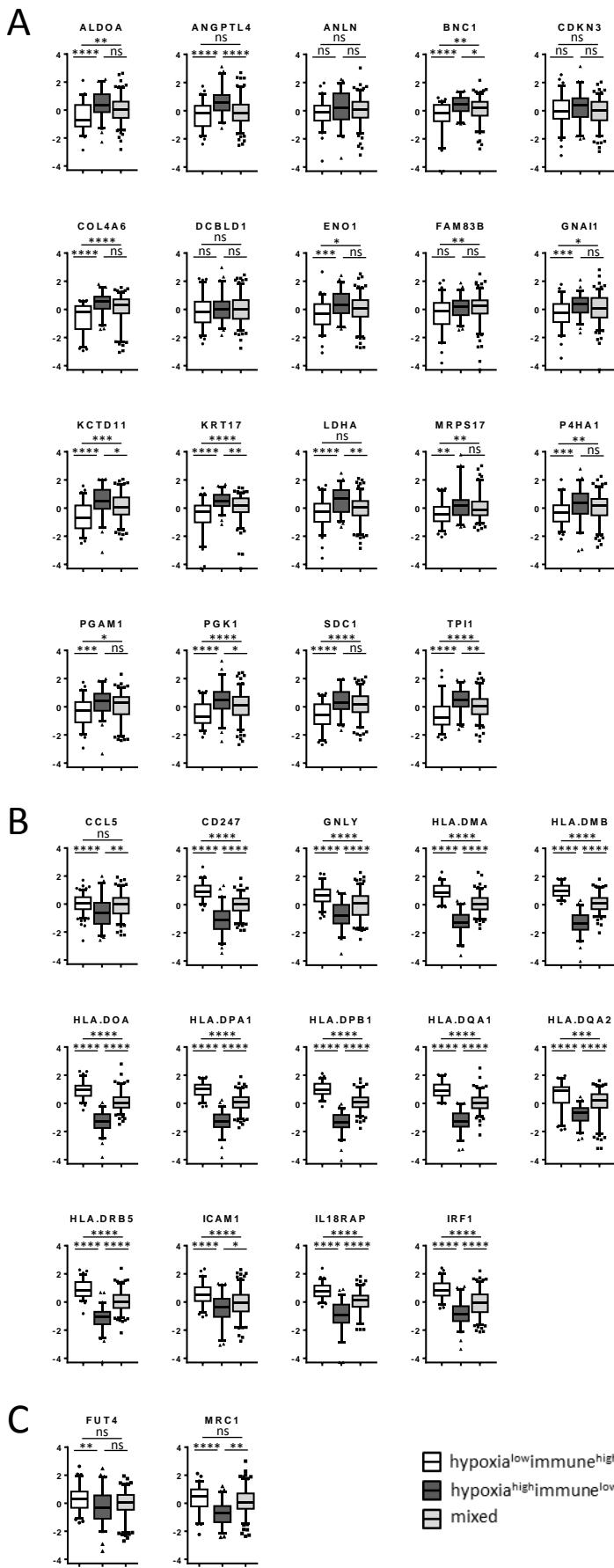


Figure S4. Relationship between heatmap defined hypoxia-immune subgroups and T stage. Graphs display percentage of samples classified as T stage 1, 2, 3 or 4 belonging to the hypoxia^{low}immune^{high}, hypoxia^{high}immune^{low} and mixed subgroups, for the TCGA (left), Chicago (middle) and Correlate (right) cohorts. Data were analysed using Fisher's exact test. p values are reported as: ns non-significant * <0.05 , ** <0.005 , *** <0.0005 , **** <0.0001 .

Figure S5 Expression of individual hypoxia- or immune-response related genes within the heat-map defined subgroups. Box-and-whisker plots showing normalised z scores for (A) Eustace hypoxia, (B) CIRC immune and (C) myeloid-related genes acquired from TCGA dataset. The $\text{hypoxia}^{\text{low}}\text{immune}^{\text{high}}$, $\text{hypoxia}^{\text{high}}\text{immune}^{\text{low}}$ and mixed subgroups (as defined by two dimensional unsupervised hierarchical clustering of gene expression) are represented as white, dark grey and light grey respectively. Data were analysed using Kruskal-Wallis test with Dunn's correction; p values are reported as: ns non-significant * <0.05 , ** <0.005 , *** <0.0005 , **** <0.0001 .



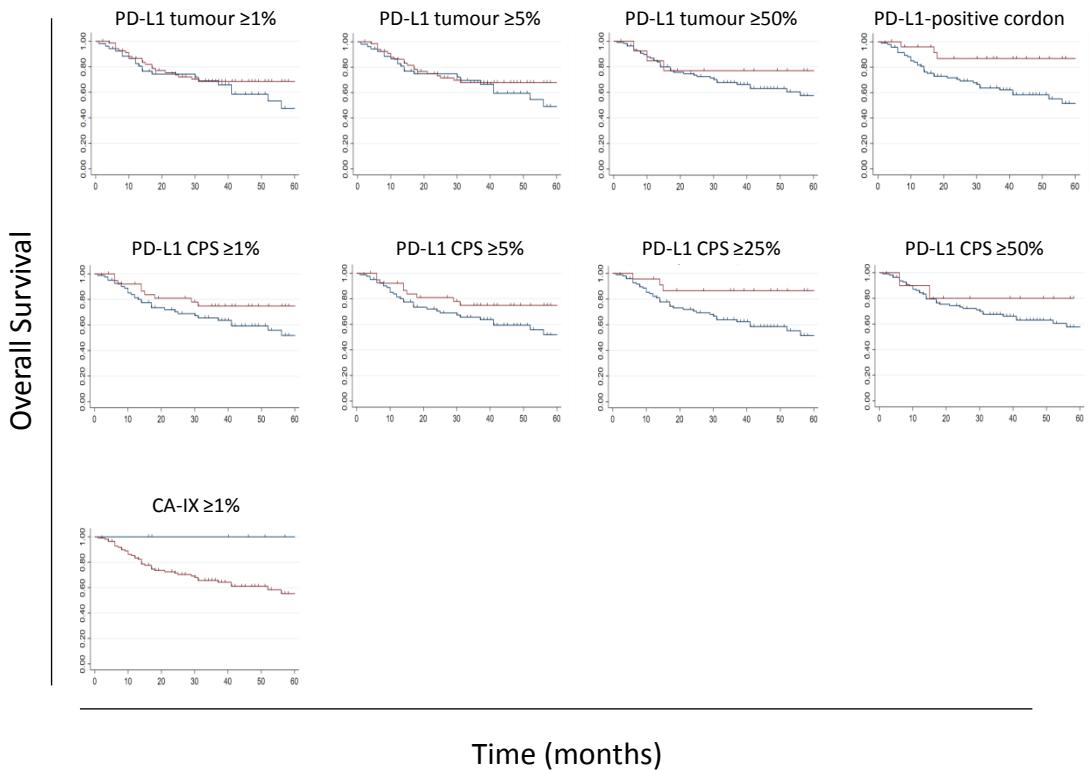


Figure S6. Correlation of PD-L1 or CA-IX protein expression with survival. Kaplan-Meier curves for OS survival relative to immune (PD-L1) or hypoxia (CA-IX) marker expression. Data are censored at 5 years.

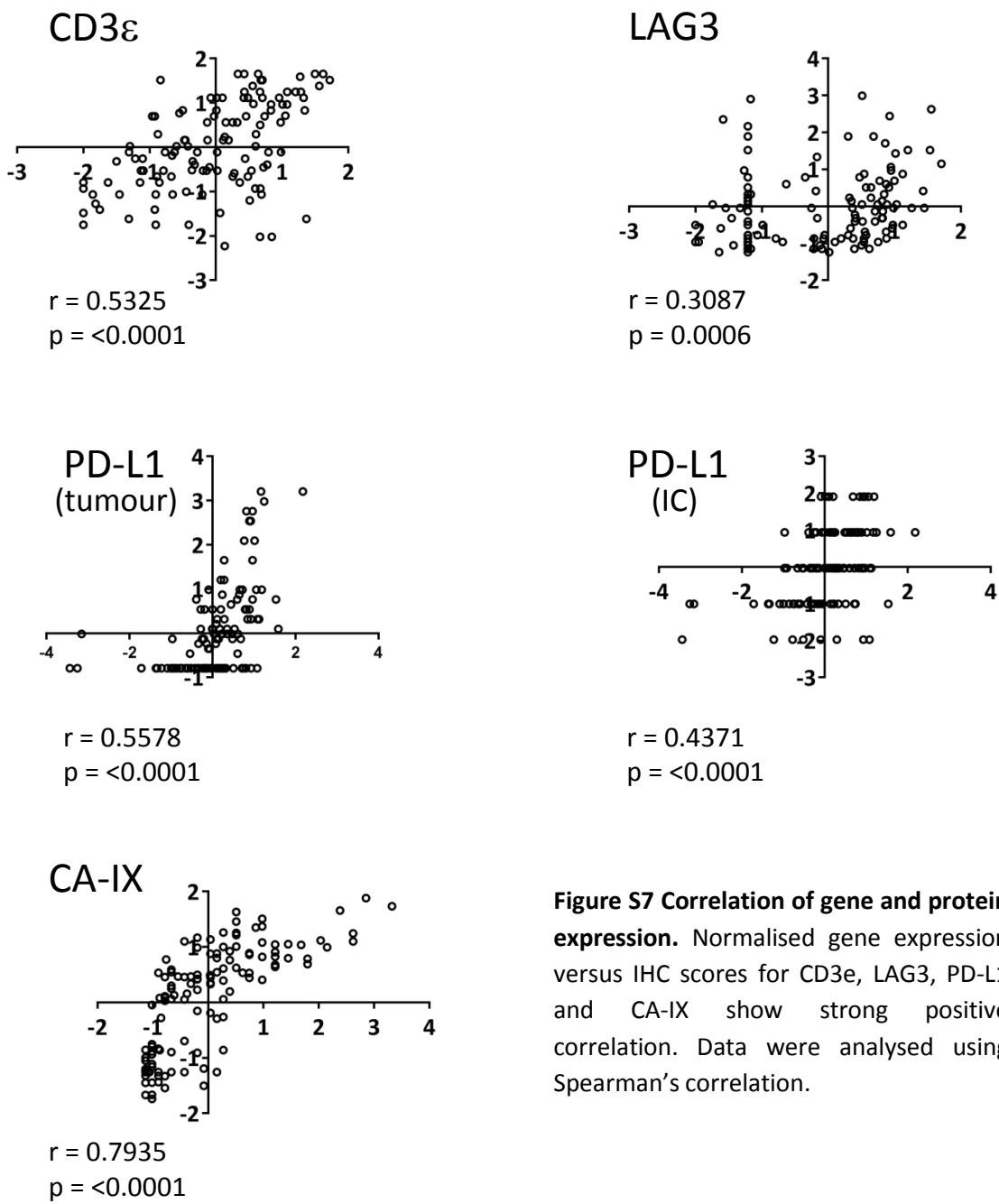


Figure S7 Correlation of gene and protein expression. Normalised gene expression versus IHC scores for CD3e, LAG3, PD-L1 and CA-IX show strong positive correlation. Data were analysed using Spearman's correlation.

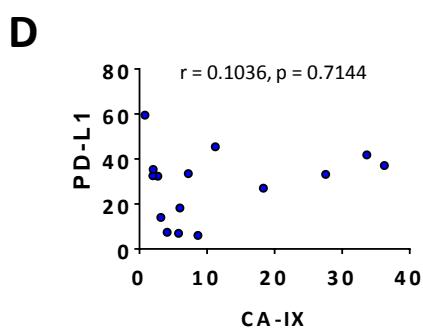
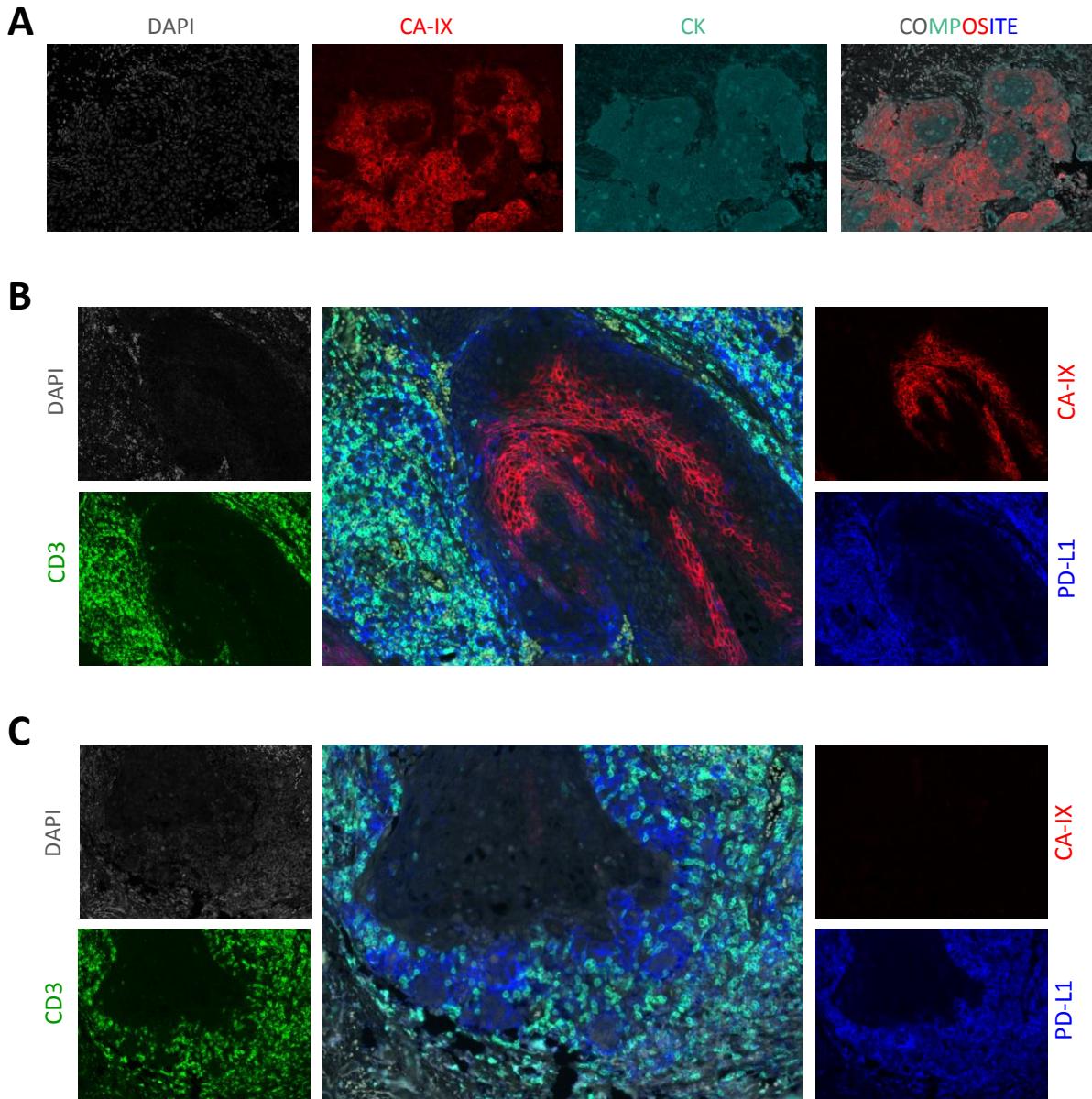


Figure S8. Spatial interactions of hypoxia and immune cells: Vectra images of multiplex stained HNC sections. (A) Co-localisation of CA-IX staining (red) with pan-cytokeratin staining (aqua) as a marker of tumour cells. (B and C) High power (20x) images from the same tumour section as in Figure 5B illustrating immune exclusion in the presence (B) and absence (C) of CA-IX. Note the presence of PD-L1-positive cells with morphology consistent with macrophage identity surrounding tumour nest. (D) InForm image analysis showed no significant correlation between CA-IX and PD-L1 expression within the tumour compartment; graph shows percentage of tumour cells positive for these markers, number of cases = 15. Data were analysed using Spearman's correlation.

Table S1 Treatment schedules***TCGA cohort***

Primary surgery + adjuvant C/RT	Primary CRT	Primary RT	Palliative C/RT	Other C/RT*	NA
73	17	3	5	13	164

* received C/RT, but schedule not specified.

NA: not available

Chicago cohort*

CRT only	Induction CT + CRT	NA
37	93	4

* all patients received organ preserving chemoradiotherapy with curative intent

Correlate cohort

Study of origin	Primary surgery + adjuvant C/RT	Primary CRT	Primary RT	Other*	NA
PET-NECK	12	21	39	1	0
PredicTR	23	41	18	7	1

* includes CT only, surgery only and none.

Patients within the PET-NECK trial were randomised to receive primary surgery + adjuvant C/RT versus primary C/RT; patients within the PredicTR study received treatment based on clinician's choice.

Table S2. Genes in the RNASeq panel/TCGA dataset

Genes in hypoxia-immune signature				Additional genes in RNASeq panel					Analysed using TCGA dataset
Eustace		CIRC		Other immune function-related		Frequently mutated in HNC		Reference control	
ALDOA	NM_0000 34.3	CCL5	NM_0012 78736.1	CD163	X_M_0052 53529.4	CASP8	NM_0010 80124.1	ACTB	NM_0011 01.3
ANGPTL4	NM_0010 39667.2	CD247	NM_0007 34.3	CD3D	NM_0007 32.4	EGFR	NM_0052 28.3		ARG1
ANLN	NM_0012 84301.2	CD274	NM_0012 67706.1	CD3E	NM_0007 33.3	NOTCH1	NM_0176 17.4		CD14
BNC1	NM_0013 01206.1	CD4	NM_0006 16.4	CD3G	NM_0000 73.2	PIK3CA	NM_0062 18.3		CD27
CA9	NM_0012 16.2	CD80	NM_0051 91.3	CD68	NM_0010 40059.1	PTEN	NM_0003 14.6		CD33
CDKN3	NM_0011 30851.1	CTLA4	NM_0010 37631.2	CD8A	NM_0011 45873.1	TP53	NM_0005 46.5		CD40
COL4A6	NM_0012 87758.1	CXCL10	NM_0015 65.3	CD8B	NM_0011 78100.1				CD163
DCBLD1	NM_1736 74.2	CXCL9	NM_0024 16.2	CDKN2A	NM_0000 77.4				ENTPD1
ENO1	NM_0012 01483.1	GNLY	NM_0013 02758.1	CIITA	NM_0002 46.3				FUT4
FAM83B	NM_0010 10872.2	HAVCR2	NM_0327 82.4	FOXP3	NM_0011 14377.1				IDO1
FOSL1	NM_0013 00844.1	HLA-DMA	NM_0061 20.3	GZMB	NM_0041 31.4				MRC1
GNAI1	NM_0012 56414.1	HLA-DMB	NM_0021 18.4	HLA-A	NM_0012 42758.1				NOS1
HILPDA	NM_0010 98786.1	HLA-DQA	NM_0021 19.3	HLA-B	NM_0055 14.7				NT5E
KCTD11	NM_0010 02914.2	HLA-DPA1	NM_0012 42524.1	HLA-C	NM_0012 43042.1				ORL1
KRT17	NM_0004 22.2	HLA-DPB1	NM_0021 21.5	IL12A	NM_0008 82.3				PRF1
LDHA	NM_0011 35239.1	HLA-DQA1	NM_0021 22.3	IL12B	NM_0021 87.2				TNFRSF4
MRGBP (C20orf2 0)	NM_0182 70.5	HLA-DQ2	NM_0200 56.4	IL18	NM_0012 43211.1				
MRPS17	NM_0159 69.2	HLA-DRA	NM_0191 11.4	IL6	NM_0006 00.4				
P4HA1	NM_0009 17.3	HLA-DRB5	NM_0021 25.3	PDCD1	NM_0050 18.2				
PGAM1	NM_0013 17079.1	ICAM1	NM_0002 01.2	PIAS1	NM_0013 20687.1				
PGK1	NM_0002 91.3	ICOS	NM_0120 92.3	STAT3	NM_0031 50.3				
SDC1	NM_0010 06946.1	IFNG	NM_0006 19.2	TNFRSF9	X_M_0115 41386.2				
SLC16A1	NM_0011 66496.1	IL18RAP	NM_0038 53.3						
SLC2A1	NM_0065 16.2	IRF1	NM_0021 98.2						
TPI1	NM_0003 65.5	LAG3	NM_0022 86.5						
VEGFA	NM_0010 25366.2	PDCD1LG2	NM_0252 39.3	STAT1	NM_0073 15.3				
				TBX21	NM_0133 51.1				

Table S3. Relationship of hypoxia-immune classifier to other prognostic variables

	TCGA	Chicago	Correlate
Hypoxia-immune gene classifier	10.08 on 1 df, p=0.0015	6.32 on 1 df, p=0.012	2.76 on 1 df, p=0.097
Age	2.18 on 1 df, p=0.1402	1.09 on 1 df, p=0.2964	3.26 on 1 df, p=0.07
Gender	3.5 on 1 df, p=0.0614	0.22 on 1 df, p=0.6395	0.85 on 1 df, p=0.3553
Tobacco use	1.73 on 1 df, p=0.1885	1.37 on 1 df, p=0.2419	0.68 on 1 df, p=0.4083
Alcohol consumption	0.15 on 1 df, p=0.6993	2.93 on 1 df, p=0.09	2.55 on 1 df, p=0.1101
T-stage	0.2 on 1 df, p=0.6583	0.83 on 1 df, p=0.3628	2.39 on 1 df, p=0.122
N-stage	2.17 on 1 df, p=0.1408	0.07 on 1 df, p=0.7976	2.19 on 1 df, p=0.1389
Clinical stage	0.11 on 1 df, p=0.7391	0.94 on 1 df, p=0.331	0.35 on 1 df, p=0.6
HPV	6.66 on 1 df, p=0.00983	8.83 on 1 df, p=0.00296	
p16			16.14 on 1 df, p=5.868e-05
Hypoxia-immune gene classifier + age	12.02 on 2 df, p=0.002	7.72 on 2 df, p=0.02	4.24 on 2 df, p=0.1
Hypoxia-immune gene classifier + gender	13.95 on 2 df, p=9e-04	6.32 on 2 df, p=0.04	3.58 on 2 df, p=0.2
Hypoxia-immune classifier + tobacco use	10.61 on 2 df, p=0.005	8.71 on 2 df, p=0.01	3.9 on 2 df, p=0.1
Hypoxia-immune classifier + alcohol consumption	9.37 on 2 df, p=0.009	6.82 on 2 df, p=0.03	6.5 on 2 df, p=0.04
Hypoxia-immune gene classifier + T-stage	10.78 on 2 df, p=0.005	5.54 on 2 df, p=0.06	3.6 on 2 df, p=0.2
Hypoxia-immune gene classifier + N-stage	12.33 on 2 df, p=0.002	5.5 on 2 df, p=0.06	4.33 on 2 df, p=0.1
Hypoxia immune classifier + clinical stage	10.08 on 2 df, p=0.006	6.33 on 2 df, p=0.04	2.48 on 2 df, p=0.3

Table shows log-rank p-values for single prognostic variables and for the hypoxia-immune classifier adjusted for each of these clinical prognosticators. Values in bold indicate statistical significance (<0.05).

Table S4. Summary of immunohistochemistry staining

	All OPSCC n = 163 (%)	P16-positive n = 93 (%)	P16-negative n = 70 (%)	P-value*
TILs				
High	52 (31.9)	46 (49.5)	6 (8.6)	<0.0001
Low	110 (67.5)	47 (50.5)	63 (90.0)	
Not available	1 (0.6)	0 (0.0)	1 (1.4)	
CD3				
Intratumoural				
High	86 (52.8)	61 (65.6)	25 (35.7)	0.0002
Low	75 (46.0)	31 (33.3)	44 (62.9)	
Not available	2 (1.2)	1 (1.1)	1 (1.4)	
Peritumoural				
Stroma				
High	66 (40.5)	52 (55.9)	14 (20.0)	<0.0001
Low	94 (57.7)	39 (41.9)	55 (78.6)	
Not available	3 (1.8)	2 (2.2)	1 (1.4)	
Tumour margin				
High	72 (44.2)	56 (60.2)	16 (22.8)	<0.0001
Low	80 (49.1)	32 (34.4)	48 (68.6)	
Not available	11 (6.7)	5 (5.4)	6 (8.6)	
Combined				
High	74 (45.4)	59 (63.4)	15 (21.4)	<0.0001
Low	87 (53.4)	33 (35.5)	54 (77.2)	
Not available	2 (1.2)	1 (1.1)	1 (1.4)	
PD-L1				
Tumour cells				
Positive ($\geq 1\%$)	83 (50.9)	55 (59.1)	28 (40.0)	0.0126
Negative ($<1\%$)	79 (48.5)	37 (39.8)	42 (60.0)	
Positive ($\geq 5\%$)	81 (49.7)	53 (57.0)	28 (40.0)	0.0264
Negative ($<5\%$)	81 (49.7)	31 (41.9)	42 (60.0)	
Positive ($\geq 25\%$)	48 (29.5)	61 (33.3)	17 (24.3)	0.1938
Negative ($<25\%$)	114 (69.9)	36 (65.6)	53 (75.7)	
Positive ($\geq 50\%$)	17 (10.4)	82 (10.7)	7 (10.0)	0.8580
Negative ($<50\%$)	145 (90.0)	74 (88.2)	63 (90.0)	
Immune cells				
High	50 (30.7)	56 (38.7)	14 (20.0)	0.009
Low	112 (68.7)	36 (60.2)	56 (80.0)	
PD-L1⁺ cordon				
Present	30 (18.4)	68 (25.8)	6 (8.6)	0.0045
Absent	132 (81.0)	24 (73.1)	64 (91.4)	
Not available	1 (0.6)	2 (1.1)	0 (0.0)	
LAG3				
Intratumoural				
High	83 (50.9)	62 (66.7)	21 (30.0)	<0.0001
Low	73 (44.8)	29 (31.2)	44 (62.9)	
Peritumoural				
High	72 (44.2)	53 (57.0)	19 (27.2)	0.0003
Low	84 (51.5)	38 (40.9)	46 (65.7)	
Combined				
High	81 (49.7)	60 (64.5)	21 (30.0)	<0.0001
Low	75 (46.0)	31 (33.3)	44 (62.9)	
Not available	7 (4.3)	2 (2.2)	5 (7.1)	
CA-IX				
Positive ($\geq 10\%$)	109 (66.9)	65 (69.9)	44 (62.9)	0.2949
Negative	53 (32.5)	27 (29.0)	26 (37.1)	
Positive ($>1\%$)	144 (88.4)	85 (91.4)	60 (85.7)	0.1696
Negative	18 (11.0)	7 (7.5)	10 (14.3)	
Not available	1 (0.6)	1 (1.1)	0 (0.0)	

*comparison of immune/hypoxia marker expression p16-positive versus negative cases (Fisher's exact test)

Table S5. Summary of statistical analyses for IHC staining

Variable	Cut-off	HR (CI)	p value	HR (CI)*	p value*
TILs	1 + 2 vs. 3	0.27 (0.12 – 0.66)	0.004	0.53 (0.2 – 1.43)	0.208
CD3					
Intratumoural	≤2.3 vs. >2.3	0.33 (0.16 – 0.67)	0.002	0.43 (0.19 – 0.97)	0.043
Peritumoural stroma	≤2.7 vs. >2.7	0.40 (0.21 – 0.77)	0.006	0.51 (0.24 – 1.08)	0.078
Tumour margin	≤2.7 vs. >2.7	0.63 (0.34 – 1.17)	0.146	1.23 (0.58 – 2.65)	0.589
Combined	≤2.6 vs. > 2.6	0.39 (0.20 – 0.77)	0.007	0.70 (0.32 – 1.53)	0.366
PD-L1					
Tumour cells	≥1%	0.71 (0.38 – 1.31)	0.273	0.78 (0.38 – 1.60)	0.493
	≥5%	0.75 (0.30 – 1.39)	0.354	0.80 (0.39 – 1.64)	0.537
	≥25%	0.39 (0.17 – 0.89)	0.025	0.33 (0.13 – 0.87)	0.025
	≥50%	0.63 (0.19 – 2.04)	0.441	0.63 (0.19 – 2.12)	0.458
Immune cells combined	≤1 vs. >1	0.55 (0.27 – 1.12)	0.101	0.45 (0.19 – 1.05)	0.066
Combined positive score	≥1%	0.54 (0.26 – 1.14)	0.105	0.41 (0.17 – 0.99)	0.048
	≥5%	0.54 (0.26 – 1.14)	0.105	0.41 (0.17 – 0.99)	0.048
	≥25%	0.27 (0.08 – 0.86)	0.028	0.09 (0.02 – 0.45)	0.004
	≥50%	0.53 (0.13 – 2.16)	0.368	0.43 (0.10 – 1.85)	0.255
Cordon	No vs. yes	0.25 (0.08 – 0.82)	0.022	0.19 (0.05 – 0.68)	0.011
LAG3					
Intratumoural	≥10%	0.60 (0.32 – 1.12)	0.106	0.94 (0.46 – 1.93)	0.866
	≥25%	0.34 (0.12 – 0.96)	0.042	0.46 (0.15 – 1.43)	0.179
Peritumoural	≥10%	0.67 (0.36 – 1.27)	0.219	0.77 (0.37 – 1.63)	0.499
	≥25%	0.19 (0.03 – 1.39)	0.103	0.22 (0.03 – 1.74)	0.151
Combined	≥10%	0.53 (0.28 – 1.00)	0.051	0.66 (0.32 – 1.36)	0.261
	≥25%	0.30 (0.07 – 1.25)	0.099	0.30 (0.06 – 1.52)	0.147
CA-IX					
	≥10%	1.23 (0.62 – 2.41)	0.555	1.29 (0.59 – 2.84)	0.521
	≥25%	1.10 (0.59 – 2.04)	0.774	0.79 (0.40 – 1.57)	0.501
	≥50%	0.99 (0.41 – 2.35)	0.977	0.66 (0.26 – 1.70)	0.387

* adjusted for age, gender, T stage, N stage, p16 status, smoking status and alcohol consumption