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# CD8+ tumour-infiltrating lymphocytes in relation to HPV status and clinical outcome in patients with head and neck cancer after postoperative chemoradiotherapy: A multicentre study of the German cancer consortium radiation oncology group (DKTK-ROG)

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**We examined the prognostic value of tumour-infiltrating lymphocytes (TILs) in patients with squamous cell carcinoma of the head and neck (SCCHN) after surgery and postoperative cisplatin-based chemoradiotherapy. FFPE-tissue originating from the surgery of 161 patients treated in 8 DKTK partner sites was immunohistochemically stained for CD3 and CD8. Their expression was correlated with clinicopathological characteristics as well as overall survival (OS), local progression-free survival (LPFS) and distant metastases free-survival (DMFS), also in the context of the HPV16-DNA/p16 status. After a median follow-up of 48 months (range: 4100 months), OS at 4 years was 46.5% for the entire cohort. In multivariate analysis, high CD8 expression was confirmed as an independent prognostic parameter for OS ($p = 0.002$), LPFS ($p = 0.004$) and DMFS ($p = 0.006$), while CD3 expression lacked significance. In multivariate analysis HPV16 DNA positivity was associated with improved OS ($p = 0.025$)**

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and LPFS ($p = 0.013$) and $p16$-positive patients showed improved DMFS ($p = 0.008$). Interestingly, high CD8 expression was a prognostic parameter for the clinical outcome in both HPV16 DNA-positive and HPV16 DNA-negative patients. Similar findings were observed in the multivariate analysis for the combined HPV16 DNA/p16 status. Altogether, CD8+ TILs constitute an independent prognostic marker in SCCHN patients treated with adjuvant chemoradiotherapy. These data indicate that CD8-positive TILs have antitumour activity and could be used for treatment stratification. Further validation of the prognostic value of CD8+ TILs as a biomarker and its role in the immune response in SCCHN patients after adjuvant chemoradiotherapy is warranted and will be performed in the prospective DKTK-ROG study.

**What's new?**

**Squamous cell carcinomas of the head and neck (SCCHN) are not a homogenous group of tumors. This means that biomarkers are urgently needed, so that prognosis and treatment can be individualised. In this study, the authors found that patients with higher levels of CD8+ tumor-infiltrating lymphocytes (TILs) within their tumors had improved outcomes after treatment. These results suggest that CD8-positive TILs may have antitumor activity, and that their expression may be a useful prognostic biomarker for treatment stratification.**



Squamous cell carcinoma of the head and neck (SCCHN) represents about 6% of the total cancer incidence and approximately 500,000 new cases are diagnosed each year worldwide.[1] Radical surgery including resection of the primary tumour and neck dissection of regional lymph nodes followed by postoperative chemoradiotherapy (CRT) is commonly performed in locally advanced SCCHN with a 5-year survival rate of 40-60%.[2,3]

The overall incidence of SCCHN is rising, especially in younger individuals due to the rising incidence in human papilloma virus (HPV)-infection-related oropharyngeal cancer.[4] HPV, mainly type 16, is encountered in approximately 25% of the patients with SCCHN and HPV-positive tumours present distinct clinicopathological features.[5] Importantly, patients with HPV-positive tumours have a survival advantage over HPV-negative SCCHN-patients after definitive CRT.[6,7] Similar findings were recently reported by our DKTK-ROG study after surgery and adjuvant CRT.[8] Although several biomarkers have been proposed in the large biologically inhomogeneous group of HPV-negative SCCHN, there is currently no molecular target that is reliable and druggable at the same time. Besides, Ang *et al.*[6] could demonstrate that there exist subgroups of HPV-positive patients with diverging prognosis. Hence, additional biomarkers are needed together with HPV status to better individualise treatment.

Malignancies are often characterized by a highly suppressive microenvironment that impairs T-cell function.[9] On the other hand, during the last decade, it has become increasingly evident that radiation therapy can induce antitumour immune responses when applied in multimodal settings.[10] The level of cluster of differentiation 3-positive (CD3+) and CD8+ tumour infiltrating lymphocytes (TILs) has been correlated with prognosis in several malignancies.[11–14] In a monocentric analysis we showed that SCCHN patients with high CD3+ and CD8+ TILs expression had improved outcomes after treatment with definitive CRT as compared with patients with low TILs infiltration.[15] Although previous studies have investigated the prognostic impact of TILs in the adjuvant setting, the majority of the reports were characterized by small sample size and/or heterogeneous postoperative treatment regimens.[16–19] Also, the impact of the immune TILs response in HPV-related SCCHN is less well investigated. In this multicentric cohort we aimed to evaluate the prognostic significance of CD3+ and CD8+ TILs alone and also in correlation with the HPV status in a larger number of patients treated homogeneously with postoperative, cisplatin-based CRT.

## Patients and Methods

### Patients and treatment

Patients were treated between 2004 and 2012 with surgery and postoperative cisplatin-based CRT in 8 DKTK partner sites (Supporting Information Table 1).[8] In all cases a squamous cell carcinoma of either the oro-, hypopharynx or oral cavity has been histologically confirmed and patients received radical surgery with neck dissection. Adjuvant CRT of the former tumour region and the regional lymph nodes was performed in patients meeting the following high risk criteria for recurrence: extracapsular spread and/or positive resection margins and/or pT4-stage and $\geq$4 positive lymph nodes. Radiotherapy consisted of elective irradiation of cervical nodes with a median dose of 50.4 Gy and a boost to the former tumour region and/or residual disease up to a total dose of 6066 Gy. Thermoplastic masks were used for immobilization and the treatment was applied by linear accelerators with energies $\geq$6 MeV using 3D-computer based or IMRT-planning. The median cumulative cisplatin dose was 200 mg/m$^2$. CT-, MRI- or PET-CT-scans were used for treatment planning and for evaluation of response. Patients without evidence of disease progression were followed-up for a minimum of 24 months. In total, $n = 221$ patients that fulfilled all criteria were included

in the study. Formalin-fixed, paraffin-embedded (FFPE) tissue originating from the surgical tumour resections was obtained. These specimens were centrally acquired together with clinical data, radiotherapy plans and diagnostic images in the DKTK RadPlanBio Platform at the DKTK partner site Dresden. From the 221 cases, sufficient FFPE material for analysis of TILs expression in all three tumour compartments (intraepithelial, stroma and tumour periphery) was available in 161 cases. A brief summary of the patient numbers sorted by treating hospital and disease site can be found in Supporting Information Table 1. This trial was approved by the ethical committees of all 8 DKTK partner sites.

### Immunohistochemical staining and scoring of CD3 and CD8

For the immunohistochemical staining of CD3 and CD8 a horseradish-peroxidase technique and a DAKO Autostainer Link 48 (DAKO, Hamburg, Germany) were used at the Department of Pathology, University Hospital Frankfurt as previously described.[20] In brief, antigen retrieval was accomplished by the pretreatment of the paraffin sections (SuperFrost Plus, Thermo Scientific, Germany) applying an Epitope Retrieval Solution (Trilog, Cell Marque, Rocklin, CA) for 30 min. Subsequently, staining was performed using standardized Dako EnVision FLEX Peroxidase Blocking reagent (K800, DAKO) and polyclonal antibodies for CD3 or CD8 (dilution 1 : 50; Dako) following incubation for 120 min at room temperature. Next, dextran polymer-conjugated horseradish-peroxidase and 3,3′-diaminobenzidine (DAB) chromogen were utilized for visualization followed by counterstaining with hematoxylin solution (Gill 3, Sigma). Sections without primary antibodies served as a negative control for both stainings. Scoring for TILs expression was conducted semiquantitatively through measuring the densities of CD3+ and CD8+ cells as previously described.[20,21] TILs were measured in three distinct compartments of the tumour: the intraepithelial compartment (cells within tumour cell nests); the stroma (cells within the intratumoural stroma) and the tumour periphery (cells localised in tumour periphery). Three random fields (×10 magnification) were examined, whereas necrotic areas were excluded. Blinded samples were evaluated by two observers (P.B. and F.R.). In case of discrepancy, a final decision was made upon further examination of the slides in a microscope based on consensus by the investigators. The total score for CD3 and CD8 was determined as the sum of the separate scores from the three tumour compartments (intraepithelial compartment, stroma and tumour periphery). The total score ranged from 3 to 12, and the median value was used as a cutoff point in order to classify patients into two groups: low or high CD3+ or CD8+ expressions. Furthermore, we examined the prognostic impact of the TIL score for each of the three different tumour areas (intraepithelial compartment, stroma and tumour periphery). For that purpose, the median TILs score of each area was calculated and the cutoff point was used to divide the cohort into subgroups with either low or high TILs score. Images were acquired using ×20 and ×40 magnification. Similar methods have been previously described for the assessment of TILs[12,13] but a shortcoming of these nonautomated systems, including our present work, is the lack of standardization in the scoring system.

### HPV16 DNA, p16 and p53 analysis

Both HPV DNA status and p16-staining were performed centrally at the DKTK partner site Dresden.[8] Immunohistochemical staining for p16 was performed using the CINtec® Histology Kit (Roche laboratories, CH). Samples with ≥70% staining were considered positive for p16.[6] The scoring was performed by two independent observers. HPV DNA analyses including genotyping were carried out using the LCD-Array HPV 3.5 Kit (CHIPRON GmbH, Berlin, DE) as described previously [8] following extraction of genomic DNA from 5 µm FFPE-sections using the QIAamp DNA FFPE tissue kit (Qiagen GmbH, Hilden, DE) based on the manufacturer's instruction. Briefly, PCR was conducted with the Primer Mix A (My 11/09) and B ('125') and the HotStarTaq Plus Master Mix (Qiagen GmbH) followed by adding hybridisation mix. The hybridisation spots were scanned and analysed using the SlideReader Software (CHIPRON GmbH), and internal quality control were also used. We investigated the prognostic value of the CD3 and CD8 in relation to the HPV16 DNA status, the p16 status and the combined HPV16 DNA/p16 status. p53 staining and scoring were performed at the DKTK partner site Dresden as recently described.[8]

### Statistics

Differences between categorical variables were evaluated using Fisher's exact test. Overall survival (OS), distant metastasis free survival (DMFS) and local progression free survival (LPFS) were calculated from the date of beginning of CRT to the day of death, distant metastasis or death and local progress or death, respectively. Patients without tumour recurrence were censored at the last follow-up contact. A $p < 0.05$ was considered statistically significant. Survival curves were plotted according to the Kaplan–Meier method using SPSS 20 for Windows (SPSS Inc., Chicago, IL). Univariate analyses were performed using the log-rank (Mantel–Cox) test and multivariate analyses by means of the Cox proportional hazard model.

## Results

### TILs staining characteristics

As a dichotomous variable, CD3 expression was defined as being "low" (weighted score <6) in 94 patients (58.4%) and "high" (weighted score ≥ 6) in 67 patients (41.6%), according to the median score. In a similar manner, CD8 expression was defined as "low" (weighted score < 6) in 96 patients (59.6%) and "high" (weighted score ≥ 6) in 65 patients (40.4%). Tumours with a high CD3 and CD8 expression were significantly associated with oropharyngeal localization, early T-stage, positive HPV16 DNA and p16 status, and lower incidence of smoking history (Table 1). Interestingly, high CD8

**Table 1.** Clinicopathological characteristics

| | Low CD3, *n* | High CD3, *n* | *p* values | Low CD8, *n* | High CD8, *n* | *p* values |
|---|---|---|---|---|---|---|
| **Age** | | | | | | |
| <Median (57 years) | 50 (52.6%) | 27 (40.9%) | 0.153 | 55 (57.3%) | 22 (33.8%) | **0.004** |
| ≥Median | 45 (47.4%) | 39 (59.1%) | | 41 (42.7%) | 43 (66.2%) | |
| **Gender** | | | | | | |
| Male | 79 (83.2%) | 52 (78.8%) | 0.540 | 81 (84.4%) | 50 (76.9%) | 0.302 |
| Female | 16 (16.8%) | 14 (21.2%) | | 15 (15.6%) | 15 (23.1%) | |
| **Tumour site** | | | | | | |
| Oral cavity | 32 (33.7%) | 9 (13.7%) | **0.008** | 32 (33.3%) | 9 (13.8%) | **0.001** |
| Oropharynx | 49 (51.6%) | 49 (74.2%) | | 47 (49%) | 51 (78.5%) | |
| Hypopharynx | 14 (14.7%) | 8 (12.1%) | | 17 (17.7%) | 5 (7.7%) | |
| **pT-staging** | | | | | | |
| pT1-2 | 50 (52.6%) | 49 (74.2%) | **0.008** | 51 (53.1%) | 48 (73.8%) | **0.009** |
| pT3-4 | 45 (47.4%) | 17 (25.8%) | | 45 (46.9%) | 17 (26.2%) | |
| **pN-staging** | | | | | | |
| pN0-1 | 26 (27.4%) | 11 (16.7%) | 0.130 | 27 (28.1%) | 10 (15.4%) | 0.085 |
| pN2-3 | 69 (72.6%) | 55 (83.3%) | | 69 (71.9%) | 55 (84.6%) | |
| **Grading** | | | | | | |
| G1 | 3 (3.2%) | 1 (1.5%) | 0.075 | 1 (1%) | 3 (4.6%) | 0.139 |
| G2 | 57 (60%) | 28 (42.4%) | | 56 (58.4%) | 29 (44.6%) | |
| G3 | 35 (36.8%) | 37 (56.1%) | | 39 (40.6%) | 33 (50.8%) | |
| **Resection margins** | | | | | | |
| R0 | 52 (54.7%) | 36 (54.5%) | 0.967 | 49 (51%) | 39 (60%) | 0.489 |
| R1 | 43 (45.3%) | 30 (45.5%) | | 47 (49%) | 26 (40%) | |
| **Extracapsular extension** | | | | | | |
| No | 43 (45.3%) | 29 (43.9%) | 0.874 | 46 (47.9%) | 26 (40%) | 0.337 |
| Yes | 52 (54.7%) | 37 (56.1%) | | 50 (52.1%) | 39 (60%) | |
| **HPV16 – DNA** | | | | | | |
| Negative | 67 (70.5%) | 33 (50%) | **0.013** | 73 (76%) | 27 (41.5%) | **<0.001** |
| Positive | 28 (29.5%) | 33 (50%) | | 23 (24%) | 38 (58.5%) | |
| **p16** | | | | | | |
| Negative | 67 (70.5%) | 27 (40.9%) | **<0.001** | 74 (77.1%) | 20 (30.8%) | **<0.001** |
| Positive | 28 (29.5%) | 39 (59.1%) | | 22 (22.9%) | 45 (69.2%) | |
| **p53** | | | | | | |
| Low | 54 (56.8%) | 45 (68.2%) | 0.188 | 54 (56.2%) | 45 (69.2%) | 0.103 |
| High | 41 (43.2%) | 21 (31.8%) | | 42 (43.8%) | 20 (30.8%) | |
| **Smoking history** | | | | | | |
| Yes | 90 (94.7%) | 56 (84.8%) | **0.051** | 92 (95.8%) | 54 (83.1%) | **0.011** |
| No (never smoked) | 5 (5.3%) | 10 (15.2%) | | 4 (4.2%) | 11 (16.9%) | |

Score was based on the median value of TILs expression; significant results have been marked with bold.

expression also correlated with an age > median (57 years). We failed to identify any further significant relationship between TILs' expression and clinicopathologic parameters (Table 1). Representative examples of low and high intraepithelial CD3 and CD8 expression are shown in Figure 1.

### TILs immunostaining and treatment response

After a median mean follow-up of 48 months (range, 4–100 months), OS at 4 years was 46.5% for the entire patient cohort. Patients with high CD3 expression had a significantly superior OS (low *vs.* high CD3: mean 64.0 *vs.* 81.4 months;

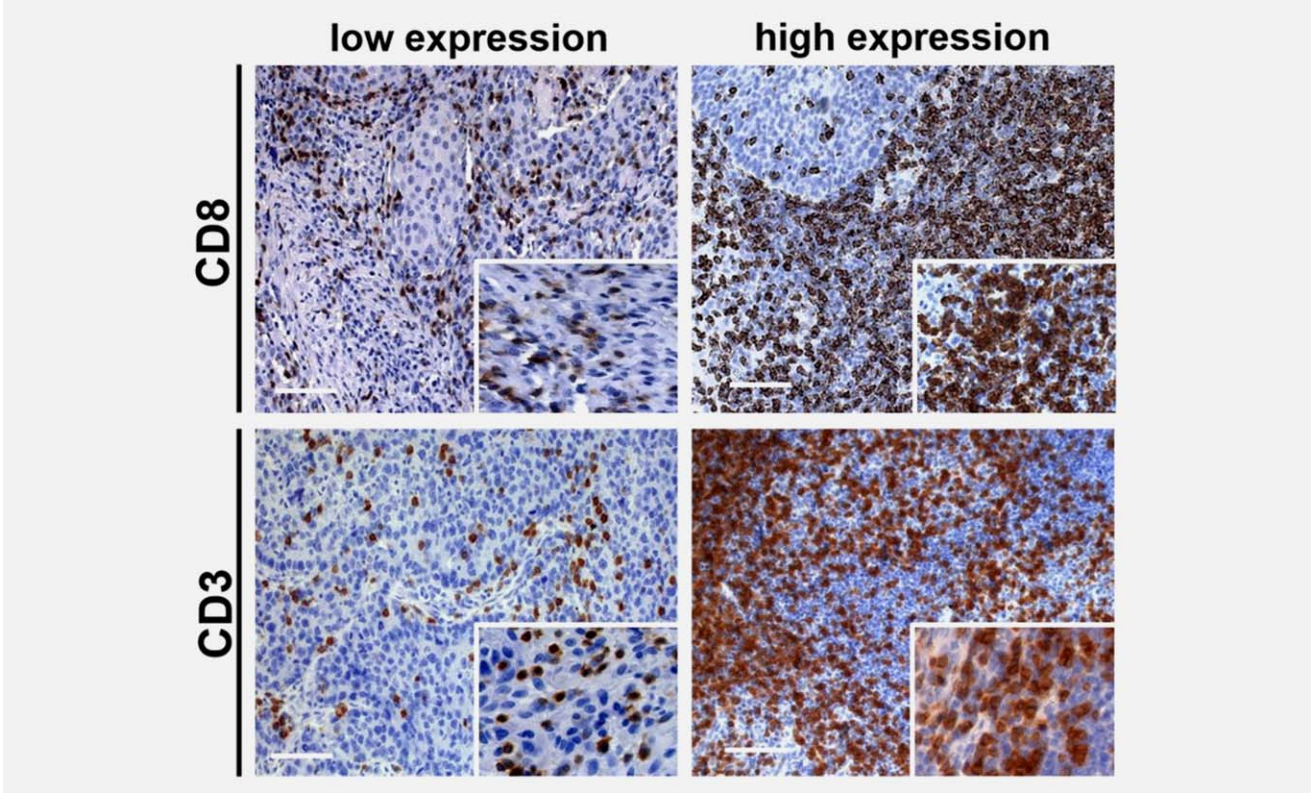


**Figure 1.** Representative examples of low and high CD8 and CD3 expression in resected squamous cell carcinoma of head and neck samples, as indicated. Original magnification, ×200 and ×400 (insert). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

$p = 0.003$), LPFS (low *vs.* high CD3: mean 57.9 *vs.* 77.7 months; $p = 0.003$) and DMFS (low *vs.* high CD3: mean 59.2 *vs.* 79.8 months; $p = 0.002$) in univariate analysis (Fig. 2(*a*) and Table 2). Similarly, patients with a high CD8 expression were characterized by a significantly superior OS (low *vs.* high CD8: mean 59.7 *vs.* 87.1 months; $p < 0.001$), LPFS (low *vs.* high CD8: mean 54.0 *vs.* 83.2 months; $p < 0.001$) and DMFS (low *vs.* high CD8: mean 55.8 *vs.* 84.5 months; $p < 0.001$; Fig. 2(*b*) and Table 2). Tumour localization (oropharynx *vs.* all other sites) significantly affected OS ($p = 0.034$) and presented a trend towards significance for LPFS ($p = 0.054$), both in favor of oropharyngeal disease site, whereas patients with extracapsular extension (ECE) had a higher incidence of distant metastases (DMFS: $p = 0.026$). Univariate analyses also revealed a significant impact for HPV16 DNA, p16 positivity and advanced T-stage on all three clinical endpoints (Table 2).

We performed a multivariate analysis by including the two TILs markers and the clinicopathological factors (Table 2). In the Cox model, high CD8 expression was confirmed as an independent prognostic parameter for OS ($p = 0.002$), LPFS ($p = 0.004$) and DMFS ($p = 0.006$) whereas no significance was found for CD3. Similarly, ECE was associated with worse OS ($p = 0.009$), LPFS ($p = 0.024$) and DMFS ($p = 0.005$). Late T-stage correlated with worse LPFS ($p = 0.038$) and DMFS ($p = 0.020$). As expected, patients negative for HPV16 DNA had adverse clinical outcome (OS: $p = 0.025$; LPFS: $p = 0.013$), whereas p16-negative patients had a higher incidence of distant metastases (DMFS: $p = 0.008$).

Furthermore, we examined the prognostic significance of TILs based on the three separate tumour compartments (tumour periphery, tumour stroma and tumour cells; Supporting Information Table 2). Intriguingly, high CD3 and CD8 score in all three compartments predicted for better outcome in our analysis.

### Correlation between TILs infiltration and HPV status

We investigated the prognostic value of the two TILs markers according to the HPV16 DNA and p16 status. Patients with positive HPV16 DNA status presented a significantly improved outcome compared to patients with HPV16 DNA negative status in univariate analysis (OS: $p < 0.001$; LPFS $p < 0.001$ and DMFS: $p < 0.001$; Table 2). In patients with HPV16 DNA-positive tumours, a high CD8 expression was a strong prognostic parameter for OS ($p = 0.023$), LPFS ($p = 0.049$) and DMFS ($p = 0.027$) but we failed to detect significance for CD3 expression with regard to the clinical endpoints (Table 3; Supporting Information Fig. 12). In patients with HPV16 DNA-negative tumours, a high CD8 expression was a strong prognostic parameter for OS ($p = 0.014$), LPFS ($p = 0.016$) and DMFS

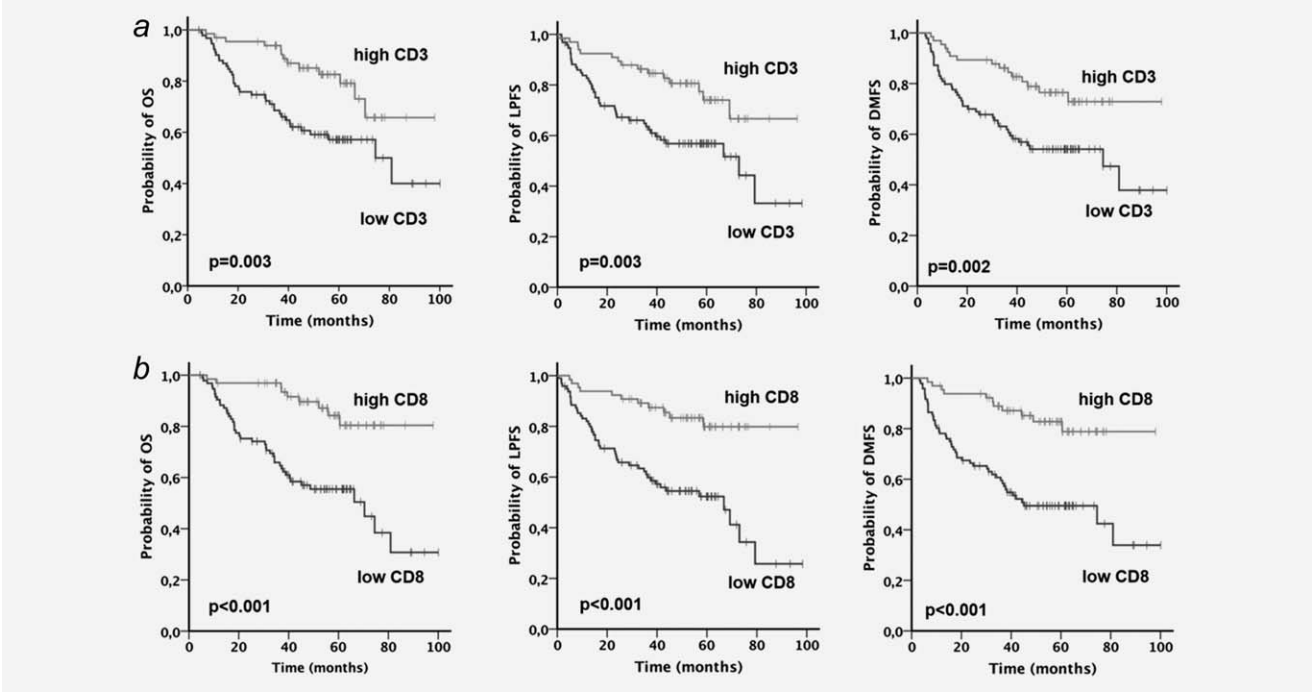


**Figure 2.** Prognostic impact of *(a)* CD8 and *(b)* CD3 on overall survival (OS), local progression-free survival (LPFS) and distant metastases free survival (DMFS) in patients with squamous cell carcinoma of head and neck after adjuvant chemoradiotherapy, as indicated. Analysis was based on the CD3 and CD8 expression in resected patient samples (low CD8 and CD3 expression: weighted score $< 6$; high CD3 and CD8 expression: weighted score $\geq 6$; cutoff-score according to median value).

($p = 0.018$). High CD3 expression also showed prognostic significance for OS ($p = 0.024$), LPFS ($p = 0.022$) and DMFS ($p = 0.031$) in the HPV16 DNA-negative cohort. Of note, similar results for CD8 expression were obtained in patients with p16-negative tumours, whereas only a trend toward significance was observed in the p16-positive arm (Table 3).

Finally, we evaluated the impact of HPV status by combining both HPV16 DNA and p16 together. In total, 83 patients (51.6%) were HPV16 DNA-negative p16-negative, 50 (31.1%) were HPV16 DNA-positive p16-positive, 17 (10.6%) were HPV16 DNA-negative p16-positive and 11 (6.8%) were HPV16 DNA-positive p16-negative. HPV16 DNA status was strongly correlated to the p16 status as shown by Fisher's exact test ($p < 0.001$). Of note, for the purpose of univariate and multivariate analysis using the combined HPV16 DNA/p16 status, only patients that had both HPV16 DNA and p16 positivity were considered HPV16 DNA/p16-positive (31.1% of all patients); otherwise the combined HPV16 DNA/p16 status was considered negative. In patients with combined HPV16 DNA/p16-negative tumours, high CD8 expression was associated with better OS ($p = 0.015$), LPFS ($p = 0.037$) and DMFS ($p = 0.038$; Table 3; Fig. 3). High CD3 expression showed prognostic significance for OS ($p = 0.028$) and LPFS ($p = 0.049$) but lacked significance for DMFS ($p = 0.065$) in the combined HPV16 DNA/16-negative cohort (Table 3; Supporting Information Fig. 3). Although high CD8 expression showed a trend for better OS ($p = 0.085$) and LPFS ($p = 0.074$), prognostic significance was only observed for DMFS ($p = 0.024$) in patients with combined HPV16 DNA/p16-positive tumours, whereas no significance was found for CD3 (Table 3; Fig. 3; Supporting Information Fig. 3). In the multivariate analysis for the combined HPV16 DNA/p16 status, findings were similar to the multivariate analysis for the HPV 16 DNA status and p16 status separately (Table 2).

## Discussion

Although several groups, including ours, have previously examined the impact of TILs on the clinical outcome in patients with locally advanced SCCHN after primary CRT, the prognostic value of CD3 and CD8 expression after postoperative CRT remains less well explored. In the present work, patients with strong and CD8+ expression had a significantly better clinical outcome compared to patients with low TILs infiltration. This observation was independent of clinicopathological parameters with a predictive role in this tumour type.

CD3 has been used as a pan-T cell marker and comprises a co-receptor to the T-cell receptor complex.[14] CD8 constitutes a glycoprotein heterodimer of alpha and beta chains that are covalently linked by a disulfide bond and serves as a co-receptor for the T-cell receptor.[14,22] CD8+ TILs are a crucial component of cell-mediated immunity as they produce interferon-γ upon interaction with tumour targets. In conjunction with the T-cell receptor, CD8 binds to the major histocompatibility complex class I molecule to promote the cytotoxic effect of TILs in killing tumour cells.[22] In

**Table 2.** Univariate and multivariate analyses of prognostic factors

| | | Multivariate | | | | Multivariate[1] | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | 95% CI | | | | 95% CI | | |
| | Univariate *p* values | HR | Lower | Upper | *p* values | HR | Lower | Upper | *p* values |
| **OS** | | | | | | | | | |
| CD3+ (high vs. low) | **0.003** | 0.987 | 0.449 | 2.166 | 0.859 | 1.116 | 0.501 | 2.485 | 0.788 |
| CD8+ (high *vs*. low) | **<0.001** | 0.297 | 0.139 | 0.632 | **0.002** | 0.311 | 0.121 | 0.799 | **0.015** |
| ECE (yes *vs*. no) | 0.090 | 2.192 | 1.218 | 3.944 | **0.009** | 2.303 | 1.245 | 4.258 | **0.008** |
| Resection status (R1 *vs*. R0) | 0.714 | | | | | | | | |
| Grade (G1 *vs*. G2 *vs*. G3) | 0.902 | | | | | | | | |
| pN-stage (pN2-3 *vs*. pN0-1) | 0.809 | | | | | | | | |
| pT-stage (pT3-4 *vs*. pT1-2) | **0.007** | 1.689 | 0.972 | 2.933 | 0.063 | 1.885 | 1.076 | 3.302 | **0.027** |
| Tumour localisation[2] | **0.034** | 0.809 | 0.533 | 1.228 | 0.318 | 0.776 | 0.495 | 1.217 | 0.269 |
| Age (<median(57) *vs*. ≥median) | 0.187 | | | | | | | | |
| Sex (male *vs*. female) | 0.597 | | | | | | | | |
| Smoking history (yes *vs*. no) | 0.241 | | | | | | | | |
| HPV16 DNA (+ *vs*. −) | **0.001** | 0.424 | 0.201 | 0.896 | **0.025** | | | | |
| p16 (+ *vs*. −) | **<0.001** | 0.663 | 0.306 | 1.437 | 0.348 | | | | |
| HPV16-DNA/p16 (+ *vs*. −) | **<0.001** | | | | | 0.385 | 0.148 | 1.002 | 0.051 |
| p53 (+ *vs*. −) | 0.143 | 0.711 | 0.383 | 1.321 | 0.500 | 0.826 | 0.450 | 1.516 | 0.537 |
| **LPFS** | | | | | | | | | |
| CD3+ (high *vs*. low) | **0.003** | 0.980 | 0.466 | 2.060 | 0.826 | 1.092 | 0.511 | 2.335 | 0.819 |
| CD8+ (high *vs*. low) | **<0.001** | 0.359 | 0.180 | 0.718 | **0.004** | 0.399 | 0.167 | 0.950 | **0.038** |
| ECE (yes *vs*. no) | 0.156 | 1.888 | 1.087 | 3.279 | **0.024** | 1.905 | 1.068 | 3.399 | **0.029** |
| Resection status (R1 *vs*. R0) | 0.401 | | | | | | | | |
| Grade (G1 *vs*. G2 *vs*. G3) | 0.402 | | | | | | | | |
| pN-stage (pN2-3 *vs*. pN0-1) | 0.843 | | | | | | | | |
| pT-stage (pT3-4 *vs*. pT1-2) | **0.004** | 1.749 | 1.033 | 2.964 | **0.038** | 1.915 | 1.122 | 3.267 | **0.017** |
| Tumour localisation[2] | 0.054 | 0.763 | 0.511 | 1.139 | 0.245 | 0.790 | 0.515 | 1.214 | 0.282 |
| Age (<median(57) *vs*. ≥median) | 0.161 | | | | | | | | |
| Sex (male *vs*. female) | 0.129 | | | | | | | | |
| Smoking history (yes *vs*. no) | 0.410 | | | | | | | | |
| HPV16 DNA (+ *vs*. −) | **<0.001** | 0.405 | 0.199 | 0.825 | **0.013** | | | | |
| p16 (+ *vs*. −) | **<0.001** | 0.619 | 0.289 | 1.324 | 0.215 | | | | |
| HPV16-DNA/p16 (+ *vs*. −) | **<0.001** | | | | | 0.333 | 0.129 | 0.856 | **0.023** |
| p53 (+ *vs*. −) | **0.035** | 0.897 | 0.497 | 1.621 | 0.950 | 0.989 | 0.554 | 1.764 | 0.969 |
| **DMFS** | | | | | | | | | |
| CD3+ (high *vs*. low) | **0.002** | 0.960 | 0.465 | 1.985 | 0.962 | 1.059 | 0.500 | 2.244 | 0.881 |
| CD8+ (high *vs*. low) | **<0.001** | 0.370 | 0.183 | 0.747 | **0.006** | 0.379 | 0.161 | 0.894 | **0.027** |
| ECE (yes *vs*. no) | **0.026** | 2.231 | 1.279 | 3.893 | **0.005** | 2.390 | 1.341 | 4.261 | **0.003** |
| Resection status (R1 *vs*. R0) | 0.545 | | | | | | | | |
| Grade (G1 *vs*. G2 *vs*. G3) | 0.957 | | | | | | | | |
| pN-stage (pN2-3 *vs*. pN0-1) | 0.768 | | | | | | | | |
| pT-stage (pT3-4 *vs*. pT1-2) | **0.004** | 1.854 | 1.104 | 3.114 | **0.020** | 1.869 | 1.104 | 3.163 | **0.020** |
| Tumour localisation[2] | 0.484 | 0.991 | 0.669 | 1.009 | 1.520 | 1.067 | 0.700 | 1.626 | 0.764 |
| Age (<median(57) *vs*. ≥median) | 0.362 | | | | | | | | |

Table 2. Univariate and multivariate analyses of prognostic factors (Continued)

| | Univariate | Multivariate | | | | Multivariate[1] | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | 95% CI | | | | 95% CI | | |
| | *p* values | HR | Lower | Upper | *p* values | HR | Lower | Upper | *p* values |
| Sex (male *vs*. female) | 0.979 | | | | | | | | |
| Smoking history (yes *vs*. no) | 0.147 | | | | | | | | |
| HPV16 DNA (+ *vs*. −) | **<0.001** | 0.568 | 0.262 | 1.234 | 0.152 | | | | |
| p16 (+ *vs*. −) | **<0.001** | 0.404 | 0.206 | 0.792 | **0.008** | | | | |
| HPV16-DNA/p16 (+ *vs*. −) | **<0.001** | | | | | 0.341 | 0.140 | 0.828 | **0.018** |
| p53 (+ *vs*. −) | **0.044** | 0.895 | 0.510 | 1.569 | 0.921 | 1.017 | 0.573 | 1.807 | 0.954 |

Abbreviations: HR: hazard ratio; CI: confidence interval; OS: overall survival; LPFS: local progression-free survival; DMFS: distant metastases free survival; ECE: extracapsular extension; HPV16 DNA/p16: combined HPV16 DNA/p16 status; (+): positive; (−): negative.
Significant results have been marked with bold.
[1]Second multivariate analysis with the implementation of the combined "HPV16-DNA/p16 status."
[2]Oropharynx versus hypopharynx/oral cavity.

agreement to our findings, strong tumour infiltration by CD8+ and CD3+ TILs has been correlated with a favorable outcome in several tumour types.[12,14,21,23–25]

Strong peritumoural TILs infiltration has been observed in SCCHN patients with lower tumour stage and less invasive growth also in patients with SCCHN.[26–28] Although several groups have assessed the prognostic impact of TILs in patients with SCCHN in the adjuvant setting, the vast majority of these studies were characterized by relatively low numbers of patients and/or heterogeneous group of patients and treatment regimens that makes interpretation of the findings difficult. Indeed, in 63 patients with laryngeal cancer, Ogino *et al.*[16] reported that a high CD8+ TILs expression correlated with a superior survival. However, only 60% received surgery, whereas 40% were treated with radiotherapy. Distel *et al* [19] demonstrated better survival in patients with high CD8 expression after postoperative radiotherapy but no CRT was administered. Le *et al.*[18] and Jung *et al.*[17] revealed improved clinical outcome in SCCHN patients with high infiltration of CD3+ TILs but treatment was heterogeneous. A major strength of the present work relies on the fact that it includes a relatively large cohort of patients ($n = 161$) treated homogeneously with adjuvant cisplatin-based CRT according to well-defined inclusion criteria, as part of this multicentre consortium group.

We did not observe any tumour compartment-dependent differences in the prognostic value of CD3+ and CD8+ TILs. Mixed findings have been reported with regard to the prognostic relevance of TILs infiltration according to the tumour compartment.[12,19,21,24,28,29] These differences could be attributed to the heterogeneity in population and treatment as in some studies surgery with or without adjuvant treatment was performed, whereas in others definitive chemoradiotherapy was administered.[12,19,21,24,28,29]

TILs mediate tumour response to cancer treatments.[12,14,21,30] Stone *et al.*[31] first showed that radiosensitivity was significantly reduced in mice lacking a normal T-cell repertoire compared to immunocompetent mice. Moreover, Burnette *et al.*[32] indicated the significance of TILs in determining the efficacy of RT *via* induction of innate and adaptive immunity as depletion of CD8+ TILs profoundly reduced tumour response to treatment. The impact of host CD8+ cytotoxic TILs to radiation response has been highlighted in recent preclinical reports on the combination of radiation and immune checkpoint inhibitors, such as Toll-like receptor 7 agonists and inhibitors of the cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) or the programmed-death-1/ligand (PD-1/PD-L1).[33–36] Stimulation of TILs by these compounds resulted to dramatic responses to RT, including long-term immunity, whereas blockade of CD8+ TILs by an anti-CD8 antibody completely abrogated this therapeutic effect,[33–36] providing strong evidence on their key role in radiation response.

Although immunohistochemical studies provide insight on the immune phenotype of the disease, TILs are often dysfunctional.[37,38] Indeed, immune inhibitory signals can lead to the so-called exhaustion of CD8+ TILs in SCCHN that could impair tumour cell killing.[38,39] Whiteside and colleagues[40,41] have previously shown that the tumours promote Fas/FasL-mediated and Bax/Bcl-XL-mediated apoptosis of circulating TILs in patients with SCCHN. Also, the immune signature of apoptosis-sensitive TILs subsets, such as CD8(+) CCR7(+) T cells in the peripheral circulation, can predict recurrence in patients with SCCHN.[42] More recently, programmed cell death-1 (PD-1), an immunecheckpoint molecule, was shown to be expressed in CD8+ TILs contributing to their dysfunctionality.[43]

HPV-positive and HPV-negative tumours constitute two different disease entities with different biological and clinical characteristics.[44] In line to our recent report,[8] patients with HPV16 DNA-positive tumours had a more favorable clinical outcome. Interestingly, high expression of CD3+ and CD8+ TILs was strongly associated with better survival in the HPV16 DNA-negative cohort in our study, whereas in the HPV-positive cohort only CD8 expression retained significance. We observed similar results regarding the prognostic role of CD8+ TILs when we combined the HPV16 DNA with the p16 status. Additionally, the CD8+ TILs' prognostic

**Table 3.** Prognostic influence of HPV16 DNA and p16 status, either alone or combined, and in correlation with the TILs markers on the clinical outcome of patients

| HPV16 DNA status and TILs marker | | OS *p* values | LPFS *p* values | DMFS *p* values |
|---|---|---|---|---|
| HPV16 DNA (+) *vs.* HPV16 DNA (−) | | **<0.001** | **<0.001** | **<0.001** |
| HPV16 (+) | CD3 | 0.460 | 0.446 | 0.631 |
| | CD8 | **0.023** | **0.049** | **0.027** |
| HPV16 (−) | CD3 | **0.024** | **0.022** | **0.031** |
| | CD8 | **0.018** | **0.016** | **0.024** |
| p16 (+) *vs.* p16 (−) | | **<0.001** | **<0.001** | **<0.001** |
| p16 (+) | CD3 | 0.705 | 0.897 | 0.475 |
| | CD8 | 0.057 | 0.085 | 0.073 |
| p16 (−) | CD3 | **0.025** | **0.029** | 0.076 |
| | CD8 | **0.018** | **0.016** | **0.023** |
| HPV16 DNA/p16 (+) *vs.* HPV16 DNA/p16 (−) | | **<0.001** | **<0.001** | **<0.001** |
| HPV16 DNA/p16 (+) | CD3 | 0.949 | 0.962 | 0.665 |
| | CD8 | 0.085 | 0.074 | **0.024** |
| HPV16 DNA/p16 (−) | CD3 | **0.028** | **0.049** | 0.065 |
| | CD8 | **0.015** | **0.037** | **0.038** |

Significant values have been marked with bold.
Abbreviations: TILs: tumour-infiltrating lymphocytes; HPV: human papilloma virus; positive; HPV16 DNA/p16: combined HPV16 DNA/p16 status; (+): positive; (−): negative; OS: overall survival; LPFS: local progression-free survival; DMFS: distant metastases-free survival.

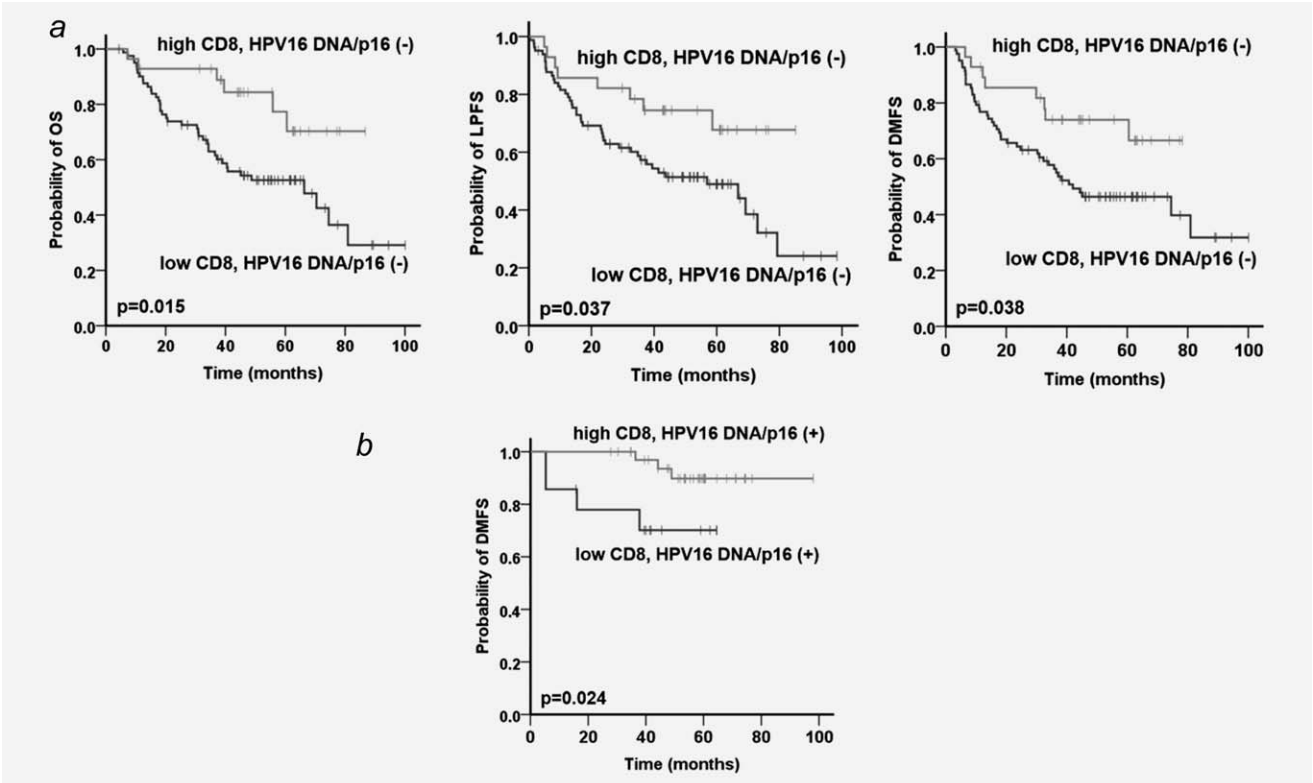


**Figure 3.** Prognostic impact of CD8 on overall survival (OS) and local progression-free survival (LPFS) and distant metastasis-free survival (DMFS) in patients with *(a)* combined HPV16 DNA/p16-negative [HPV16 DNA/p16 (−)] and *(b)* combined HPV16 DNA/p16-positive [HPV16 DNA/p16 (+)] status after adjuvant chemoradiotherapy, as indicated. Only significant results are shown.

significance was more apparent in patients with HPV-negative status compared to those with positive HPV status, especially after using the combined HPV16 DNA/p16 status (Table 3). This could be explained, at least in part, by the difference in the degree of TILs infiltration between the two subgroups. High CD8+ TILs infiltration was more common in HPV-positive compared to HPV-negative tumours in our series. The prognostic value of TILs in correlation with the HPV status in SCCHN has been the subject of recent studies. Nordfors *et al.*[45] and Nasman *et al.*[46] showed that strong intratumoural infiltration by CD8+ TILs was associated with improved clinical outcome in patients with both HPV-positive and HPV-negative carcinomas. Ward *et al.*[18] and Jung *et al.*[47] reported strong TILs infiltration in HPV-positive tumours that correlated with improved OS. A recent study conducted an integrative analysis in head and neck cancer and identified five molecular subgroups.[48] Of those, high CD8+ TILs expression in HPV-positive tumours of the inflamed/mesenchymal subgroup had better outcome compared to the second HPV-related subgroup with a different molecular phenotype. Kong *et al.* demonstrated significance for high CD3+ TILs expression only in HPV-negative tumours[49] that is similar to our previous work in the definitive CRT setting.[20] Wansom and colleagues[50] failed to detect any difference in TILs expression or prognostic impact by HPV status. The different findings could be attributed to the inhomogeneous therapeutic modalities and patient cohorts. Hence, prospective randomized trials studies are required to better elucidate the prognostic significance of TILs according to the HPV status.

We acknowledge that although prospectively treated and followed-up, the retrospective evaluation of the TILs' prognostic impact constitutes a limitation of our study and hence potential selection bias cannot be excluded. Also, the median follow-up in our study is relatively short as studies with a follow-up of several years have been previously reported.[6,51] TIL's score was assessed using a nonautomated system due to the lack of scoring system standardization that constitutes another limitation of our work. Finally, immunohistochemical analysis of TILs does not provide any information on their functionality and hence this issue should be considered when interpreting our findings.

In summary, CD8+ TILs represent a promising prognostic marker to identify SCCHN patients with a more favorable clinical outcome after adjuvant CRT. The use of CD8 as an additional marker together with HPV16 DNA to predict clinical outcome could have direct translational implications since high-risk patients could potentially benefit from novel immunotherapies to stimulate T-cell-activity in combination with CRT. Our findings warrant validation in prospective cohorts before use of CD8+ TILs as a routine biomarker in the clinical setting and will be explored in our prospective DKTK-ROG study.

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