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Prognostic Value of Total CD8+ Density Versus Spatial Distribution in Head and Neck Squamous Cell Carcinoma

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Abstract

Background

The prognostic value of CD8 + T-cell spatial distribution within the tumor microenvironment of head and neck squamous cell carcinoma (HNSCC) remains unclear. We investigated whether the ratio of CD8 + density in the tumor center to the invasion front predicts survival and compared this with total CD8 + density.

Methods

We analyzed 709 HNSCC patients from the public HANCOCK dataset with tissue microarray-based CD8 + quantification at both the tumor center and the invasion front. Center-to-front CD8 + ratios were calculated and categorized into tertiles. Total CD8 + density (center + front) was also analyzed. Kaplan-Meier, log-rank, and Cox proportional hazards models were used for survival analysis, adjusting for age, HPV status, nodal status, and pT stage.

Results

Median CD8 + density did not differ between tumor center (393.9 cells/mm²) and invasion front (344.1 cells/mm², p = 0.828). No significant survival difference was observed among ratio tertiles (log-rank Low vs High: p = 0.477; multivariate HR = 1.17, 95% CI: 0.87–1.57, p = 0.300). In contrast, higher total CD8 + density was modestly but statistically significantly associated with improved survival (HR = 0.99 per 100 cells/mm², 95% CI: 0.97-1.00, p = 0.033). Established prognostic factors were confirmed: age (HR = 1.03, p < 0.001), HPV positivity (HR = 0.52, p = 0.006), nodal positivity (HR = 1.72, p = 0.002), and pT3-4 stage (HR = 1.92, p < 0.001).

Conclusions

The center-to-front CD8 + ratio has limited prognostic value in HNSCC. However, higher total CD8 + density is associated with improved survival, suggesting that overall immune burden, rather than topographic distribution, drives clinical outcomes. These findings suggest that overall CD8 + immune burden may be more informative than spatial distribution for prognostic assessment in HNSCC

Introduction

Head and neck squamous cell carcinoma (HNSCC) remains a significant global health burden, with over 800,000 new cases and 400,000 deaths annually [1, 2]. Despite advances in multimodal therapy, including surgery, radiotherapy, and immunotherapy, prognosis remains highly heterogeneous, driving the ongoing search for reliable biomarkers to guide treatment stratification and predict clinical outcomes [3].

The tumor immune microenvironment, particularly the infiltration of CD8 + cytotoxic T lymphocytes, has emerged as a key determinant of prognosis and treatment response across multiple cancer types [4, 5]. In HNSCC, high CD8 + density generally correlates with favorable outcomes, and this association has been validated in numerous observational studies and meta-analyses, establishing CD8 + quantification as a promising prognostic tool [6–8].

However, whether the *spatial distribution* of CD8 + T cells within the tumor, specifically, the balance between CD8 + density at the tumor center and the invasive front, provides additional prognostic information beyond overall density remains contentious [9, 10]. Several small-to-moderate-sized studies have examined this question in HNSCC with conflicting results. Some reported that high CD8 + density at the invasion front predicts better survival, others found that center-derived density was more informative, and still others observed no spatial gradient effect [11, 12].

These discrepancies likely reflect important limitations of prior research: small sample sizes (typically n < 200), heterogeneous methodologies for quantifying CD8 + cells, and a lack of direct comparisons across compartments. However, few large-scale studies have examined whether spatial distribution adds independent prognostic value beyond overall CD8 + burden in HNSCC.

To address these limitations, we used the HANCOCK dataset, a large, publicly available collection of 763 HNSCC patients with tissue microarray-based quantification of CD8 + cells at both the tumor center and the invasion front. We investigated whether the center-to-front CD8 + ratio provides prognostic information independent of established clinical factors. This study is among the largest and directly compares the ratio to total density, evaluating its incremental prognostic value.

Methods

Study Population and Data Source

This retrospective analysis utilized the HANCOCK (Head and Neck Squamous Cell Carcinoma COhort for Knowledge) dataset, which is publicly available through The Cancer Imaging Archive (DOI: 10.7937/rcty-5h16) [13]. The dataset includes 763 patients with histologically confirmed HNSCC originating from four anatomical subsites: oropharynx, larynx, oral cavity, and hypopharynx. All patients were diagnosed between 2010 and 2020 and underwent surgical resection with curative intent. For the present analysis, we included only patients with complete CD8 + TMA data from both the tumor center and the invasion front, as well as complete clinical follow-up data. This yielded a final analytic cohort of 709 patients, with those having incomplete data excluded.

Tissue Microarray Construction and CD8 + Immunohistochemistry

Formalin-fixed, paraffin-embedded (FFPE) tumor blocks from surgical resection specimens were retrieved from the pathology archives of participating HANCOCK institutions. For each patient,

representative tumor center and invasive front regions were identified and annotated by two board-certified head and neck pathologists (blinded to clinical outcomes) based on review of hematoxylin and eosin (H&E)-stained whole-slide sections. The tumor center was defined as the deepest viable tumor area without surface epithelium or invasive edge, located at least 5 mm from the tumor-stromal interface. The invasive front was defined as the leading edge of tumor infiltration, including the epithelial tumor nests and immediately adjacent desmoplastic stroma within 500 μm of the tumor-stromal interface. Necrotic areas, hemorrhagic zones, and regions with processing artifacts were excluded from annotation.

Tissue microarrays (TMAs) were constructed using a manual tissue arrayer (Beecher Instruments, Sun Prairie, WI, USA) with a 1.0 mm core diameter. From each annotated region, three replicate cores were obtained to account for intratumoral heterogeneity. Separate TMA blocks were prepared for the tumor center and the invasion front compartments. For the tumor center, 184 cores were arrayed, representing regions from 184 patients (1–3 cores per patient). For the invasion front, 184 separate cores were arrayed. Interobserver agreement for compartment annotation was assessed on a pilot set of 20 cases (40 regions), yielding a Cohen's kappa of 0.87 (95% CI: 0.79–0.95), indicating excellent agreement.

Immunohistochemical staining for CD8 was performed on 4-μm TMA sections using an automated stainer (Ventana BenchMark ULTRA, Roche Diagnostics). Following heat-induced antigen retrieval (CC1 buffer, pH 8.0, 64°C for 64 minutes), sections were incubated with anti-CD8 primary antibody (clone C8/144B, Dako, 1:100 dilution, 32 minutes at 37°C). Detection was performed using the OptiView DAB IHC Detection Kit (Ventana). Positive controls (tonsil tissue) and negative controls (omission of primary antibody) were included in each run [13, 14].

Quantification of CD8 + Cell Density

Digital whole-slide images were acquired at 20× magnification (0.5 μm/pixel) using a Hamamatsu NanoZoomer S360 scanner. Automated image analysis was performed using QuPath software (version 0.5.0, University of Edinburgh). The segmentation algorithm employed the following sequential steps: (1) hematoxylin-OD color deconvolution to separate nuclear and cytoplasmic channels; (2) Gaussian filtering (sigma = 1.5 μm) for noise reduction; (3) Otsu thresholding for nuclear detection; (4) cell expansion to 10 μm to define cytoplasmic boundaries; (5) classification of CD8 positivity based on cytoplasmic DAB intensity threshold (intensity > 0.2 after normalization, validated against pathologist scoring).

Algorithm validation was performed on 100 randomly selected cores (50 center, 50 front). Automated counts were compared to manual counts by two pathologists blinded to algorithm results. Intraclass correlation coefficients were 0.94 (95% CI: 0.91–0.96) for CD8 + counts and 0.92 (95% CI: 0.88–0.95) for density estimates. The algorithm was specifically validated for HNSCC tissue architecture, including keratinizing and non-keratinizing morphologies. For each core, CD8 + cell density was calculated as the number of positive cells per mm². For patients with multiple cores from the same compartment, the mean density across cores was calculated [13, 14].

Clinical and Pathological Variables

Clinical and pathological data were extracted from the HANCOCK clinical database, which includes comprehensive patient-level information. The following variables were included in the analysis: age at diagnosis (continuous, years), sex (male or female), primary tumor site (oropharynx, larynx, oral cavity, or hypopharynx), smoking status (current smoker, former smoker, or never smoker), pT stage according to the AJCC 7th edition (categorized as pT1-2 vs pT3-4), pN stage (categorized as node-negative [pN0] vs node-positive [pN1-3]), and HPV status. HPV status was determined by p16 immunohistochemistry, with positivity defined as strong and diffuse nuclear and cytoplasmic staining in ≥ 70% of tumor cells. Overall survival (OS) was defined as the time from the date of diagnosis to the date of death from any cause, or to the last known follow-up, censored at the date of last contact for living patients.

Statistical Analysis

The center-to-front CD8 + ratio was calculated as: Ratio = (mean CD8 + density at tumor center) / (mean CD8 + density at invasive front). Due to the potential for statistical instability (amplification of measurement error, skewed distribution, division-by-small-number artifacts), we implemented several pre-specified analytical approaches:

1. **Continuous analysis**: Log-transformed ratio (ln ratio) was used in Cox regression to normalize the distribution. Extreme values (ratio > 10 or < 0.1; n = 12 patients, 1.7%) were winsorized to the 1st and 99th percentiles.
2. **Categorical analysis**: Patients were stratified by distribution-based tertiles after winsorization, with cutoffs determined by the 33.3rd and 66.7th percentiles of the empirical distribution. The reported cutoffs (1.0 and 2.0) are rounded approximations; actual cutoffs were 0.98 and 1.96.
3. **Sensitivity analysis**: Analysis was repeated excluding patients with front density < 50 cells/mm² (n = 23, 3.2%) to assess robustness to division-by-small-number artifacts.

The primary analysis used a log-transformed continuous ratio. Tertile-stratified analysis is presented for clinical interpretability.

Among 763 patients in the HANCOCK dataset, 54 (7.1%) were excluded due to missing TMA data (n = 31, missing either center or front cores), incomplete clinical follow-up (n = 18), or missing survival outcome (n = 5). Among the 709 included patients, missingness was as follows: HPV status (p16) was missing/not tested in 401 patients (56.5%); these patients were treated as a separate category in sensitivity analyses but excluded from HPV-adjusted models. Nodal status was complete for all 709 patients. The pT stage had 3 missing values (0.4%), excluded from stage-adjusted models. A complete-case analysis was performed for each multivariable model, assuming missingness-at-random, given the retrospective real-world data source. Multiple imputation was not performed due to the high proportion of missing HPV data (> 50%), which would produce unstable imputations.

Variables were selected a priori based on established prognostic factors in HNSCC from published literature and clinical guidelines. The base model included age (continuous), HPV status (positive vs negative, with negative defined as p16-negative or not tested as sensitivity), nodal status (positive vs negative), and pT stage (pT3-4 vs pT1-2). Tumor subsite was considered for inclusion but excluded from the primary model due to strong collinearity with HPV status (oropharyngeal tumors accounting for 85% of HPV-positive cases); subsite-stratified sensitivity analyses are reported in the supplementary materials. Treatment modality was not included due to heterogeneity and a lack of standardized protocol across the multi-institutional cohort. Extracapsular extension and margin status were not available in the HANCOCK dataset.

Univariable and multivariable Cox proportional hazards regression models were used to estimate hazard ratios (HRs) and 95% confidence intervals (CIs) for associations with overall survival. The multivariable model was constructed a priori to include established prognostic factors in HNSCC: age (continuous), HPV status (positive vs negative), nodal status (positive vs negative), and pT stage (pT3-4 vs pT1-2). The center-to-front ratio (High vs Low) was then added to this base model to evaluate its independent prognostic value. Total CD8 + density was evaluated in a separate model as both a categorical (High vs Low/Medium) and continuous (per 100 cells/mm²) variable. Proportional hazards assumptions were verified using Schoenfeld residuals. All statistical tests were two-sided, and p-values < 0.05 were considered statistically significant. Given the exploratory nature of the total CD8 + density analysis, no adjustment for multiple comparisons was applied, though the primary finding (center-to-front ratio) was pre-specified. Analyses were conducted using Python version 3.12 with the lifelines package for survival analysis.

Results

Patient Cohort

A total of 709 patients with HNSCC and complete CD8 + TMA data were analyzed. Median age was 61 years (range 22–92), with male predominance (79.5%). Primary tumor sites included oropharynx (43.6%), larynx (26.9%), oral cavity (18.2%), and hypopharynx (11.3%). HPV positivity (p16+) was observed in 18.5% (n = 131), nodal metastasis in 72.4% (n = 513), and pT3-4 stage in 28.3% (n = 201). During a median follow-up of 41.1 months, 193 patients (27.2%) had died. The details are shown in Table 1.

Table 1
Patient Characteristics (n = 709)

Characteristic	Category	N, (%)
Total patients		709 (100%)
Age, median (range)		61 (22–92)
Sex	Male	564 (79.5%)
	Female	145 (20.5%)
Tumor site	Oropharynx	309 (43.6%)
	Larynx	191 (26.9%)
	Oral Cavity	129 (18.2%)
	Hypopharynx	80 (11.3%)
HPV16	Positive	131 (18.5%)
	Negative	177 (25.0%)
	Not tested	401 (56.5%)
Node status	Node positive	513 (72.4%)
	Node negative	196 (27.6%)
pT stage	pT1-2 stage	505 (71.2%)
	pT3-4 stage	201 (28.4%)
	Missing	3 (0.4%)
Death events		193 (27.2%)
Median follow-up, months (IQR)		41.1 (23.5–62.8)

CD8 + Spatial Distribution and Survival

Median CD8 + density did not differ between the tumor center (393.9 cells/mm²) and the invasion front (344.1 cells/mm², p = 0.828). Patients were stratified into tertiles based on center-to-front ratio: Low (< 1.0, n = 236), Medium (1.0–2.0, n = 237), and High (> 2.0, n = 236).

No significant survival difference was observed among ratio groups (log-rank Low vs High: p = 0.477) (Fig. 1). In multivariate analysis adjusting for age, HPV status, nodal status, and pT stage, the center-to-front ratio remained non-prognostic (HR = 1.17, 95% CI: 0.87–1.57, p = 0.300).

Total CD8 + Density Predicts Survival

In contrast to spatial distribution, total CD8 + density (center + front) was significantly associated with survival. When analyzed as a continuous variable (per 100 cells/mm² increase), higher total CD8 + density predicted improved survival (HR = 0.99, 95% CI: 0.97-1.00, p = 0.033) (Fig. 2).

Independent Prognostic Factors

Multivariate Cox regression confirmed established prognostic factors: age (HR = 1.03 per year, p < 0.001), HPV positivity (HR = 0.52, p = 0.006), nodal positivity (HR = 1.72, p = 0.002), and pT3-4 stage (HR = 1.92, p < 0.001). Sex was not prognostic (p = 0.314) (Fig. 3, Table 2).

Table 2
Multivariate Cox Regression

Variable	HR (95% CI)	p-value
Age (per year)	1.03 (1.02–1.05)	< 0.001
HPV positive (vs negative)	0.52 (0.33–0.83)	0.006
Node positive (vs negative)	1.72 (1.23–2.43)	0.002
pT3-4 (vs pT1-2)	1.92 (1.43–2.59)	< 0.001
Center-to-front ratio (High vs Low)	1.17 (0.87–1.57)	0.3
Total CD8+ (per 100 cells/mm²)	0.99 (0.97-1.00)	0.033

Discussion

In this study of 709 HNSCC patients with quantitative CD8 + density measurements from both tumor center and invasion front, we make three observations. First, the center-to-front CD8 + ratio is not associated with overall survival, suggesting that spatial distribution metrics lack clinical utility. Second, total CD8 + density, when analyzed continuously, does predict improved survival, suggesting that overall immune burden matters more than topographic localization. Third, established prognostic factors; age, HPV status, nodal status, and pT stage are robustly confirmed in this large external cohort, validating the representativeness of our study population.

Our null finding for spatial distribution contrasts with several smaller studies that reported prognostic value for CD8 + density at either the invasion front or tumor center. Balermpas and colleagues reported that high CD8 + density at the invasion front was associated with better survival in 101 HNSCC patients receiving chemoradiotherapy [15]. Similarly, other studies found that high intratumoral CD8 + density (center) was associated with favorable outcomes in surgically treated patients [10, 16]. However, these studies were limited by modest sample sizes and did not include a direct head-to-head comparison between compartments. A meta-analysis by Yang et al. suggested that both compartments may have prognostic value but noted significant heterogeneity across studies, with I^2 values exceeding 50%,

indicating substantial between-study variation [17]. Our larger sample size (n = 709) provides greater statistical power to detect modest associations than prior studies.

Some biological explanations may account for the absence of a spatial gradient effect in our study. HNSCCs, particularly those arising from the oropharynx and oral cavity, sometimes exhibit relatively uniform immune infiltration patterns compared to other solid tumors, such as colorectal or lung cancer, where compartmentalization is more pronounced [18, 19]. The invasive front in HNSCC is frequently characterized by desmoplasia and stromal remodeling, which may physically impede CD8 + T-cell accumulation regardless of tumor center density [20]. The center-to-front ratio may simply be a surrogate for overall CD8 + burden; when total density is accounted for, the ratio adds no independent information [11, 21]. Our findings may suggest that broad compartmental differences are less pronounced in HNSCC than in some other tumor types.

The finding that continuous total CD8 + density predicts improved survival (HR = 0.99 per 100 cells/mm², p = 0.033) aligns with the broader literature establishing CD8 + density as a robust prognostic biomarker across multiple cancer types [4, 22]. However, the effect size is modest, approximately a 1% relative risk reduction per 100 cells/mm² increment. This is consistent with prior studies reporting hazard ratios for CD8 + density typically ranging from 0.85 to 0.95 per standard deviation increase [23, 24]. The clinical implication is that CD8 + density should be interpreted as one component of a multivariable risk model rather than a standalone binary classifier. For example, a patient with a total CD8 + density two standard deviations above the mean (approximately 1,000 cells/mm² higher than a patient with low density). This would be estimated to have a 10% lower relative risk of death and may provide incremental prognostic information when combined with established clinicopathological variables.

Our analysis shows that age (HR = 1.03 per year), HPV positivity (HR = 0.52), nodal positivity (HR = 1.72), and advanced pT stage (HR = 1.92) have independent prognostic value. These findings align with decades of clinical research and provide important validation that our cohort is representative of the broader HNSCC population [25, 26]. The fact that these established factors remained significant in multivariable models adjusting for CD8 + metrics suggests that immune markers add information to, but do not replace, traditional clinicopathological variables. Future risk stratification algorithms should integrate both clinical factors and immune biomarkers.

Clinical Implications

Our findings suggest that overall CD8 + burden may be more informative than simple center-to-front ratios in HNSCC. The absence of a spatial gradient suggests that, in HNSCC, CD8 + T cells may infiltrate tumors relatively uniformly, unlike in some other cancer types, where compartmentalization is more pronounced. Further studies are needed before incorporating detailed spatial metrics into routine pathology reporting.

Limitations

TMA-based analysis may not fully capture the whole-slide immune architecture or spatial heterogeneity at the invasive margin. HPV status was assessed by p16 immunohistochemistry rather than direct HPV testing (DNA or RNA), which may misclassify a small proportion of patients, particularly those with non-oropharyngeal HNSCC. As with any retrospective analysis, residual confounding by unmeasured variables (e.g., performance status, comorbidity, detailed smoking pack-years) cannot be excluded. The modest effect size for total CD8 + density (HR = 0.99 per 100 cells/mm²) may limit clinical utility for individual patient prediction, though it remains statistically significant and biologically plausible.

Future Directions

Prospective studies using standardized protocols to quantify CD8 + cells are needed to establish clinically meaningful cutoffs and to validate our findings in independent cohorts. Integrating CD8 + density with other immune markers (e.g., PD-L1 expression, CD4 + T cells, macrophages, tertiary lymphoid structures) may yield composite scores with greater prognostic accuracy. The interaction between CD8 + density and specific treatment modalities (immunotherapy, chemoradiation) warrants investigation to determine whether CD8 + burden predicts treatment response beyond its prognostic value. Applying deep learning to whole-slide images may enable automated, continuous CD8 + quantification that captures spatial heterogeneity more completely than TMA-based approaches.

Conclusions

This study demonstrates that the center-to-front CD8 + ratio may not have independent prognostic value. In contrast, higher total CD8 + density is associated with improved survival, suggesting that overall immune burden, rather than topographic distribution, is associated with clinical outcomes. Established prognostic factors, including age, HPV status, nodal status, and pT stage, are robustly confirmed. These findings suggest limited prognostic utility for spatial CD8 + mapping while supporting the importance of total CD8 + quantification. Routine assessment of total CD8 + density, integrated with established clinicopathological variables, may improve risk stratification in HNSCC. However, the modest effect size suggests that immune biomarkers should complement rather than replace traditional prognostic factors.

Declarations

Ethics and Consent to Participate

Not applicable (Secondary analysis of publicly available data)

Clinical trial Number

Not applicable

Consent to Publish

Not applicable

Conflict of Interest

The authors declare no competing interests.

Author Contributions

OD and EM conceived the research idea and coordinated the study. WK, EY, and EM contributed to the refining of the study design and methodology. OD, NB, and WK were actively involved in data analysis and interpretation. OD, NB, and EY were the primary contributors to the manuscript. All authors were involved in the review and approval of the final manuscript.

Data Availability

The HANCOCK dataset is publicly accessible at https://hancock.research.fau.eu/download. The dataset is distributed under a Creative Commons Attribution (CC BY) license, permitting reuse with appropriate citation. All analyses were conducted in accordance with the dataset's terms of use. No institutional review board approval was required for this secondary analysis of de-identified public data.

The complete analysis code is available on GitHub: https://github.com/oumodavid1625/Work-on-Data-Science/blob/main/Head%20and%20Neck%20Cancer%20Immunoprofiling%20Analysis.ipynb

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Figures

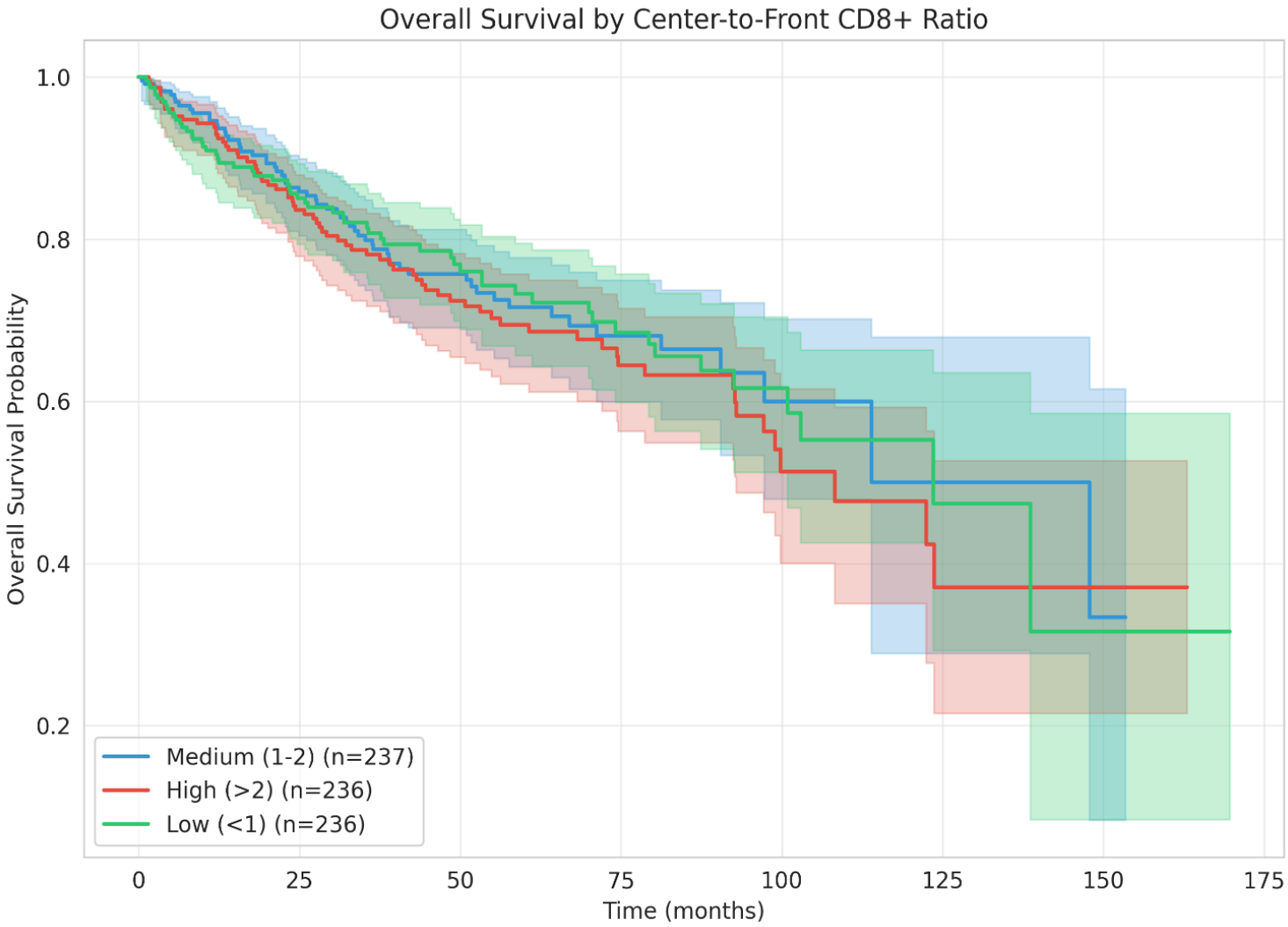


Figure 1

Kaplan-Meier Survival Analysis: Center-to-front CD8+ ratio. Patients were stratified into tertiles: Low (<1.0, n=236), Medium (1.0-2.0, n=237), and High (>2.0, n=236). No significant difference in survival was observed among the three groups (log-rank test: Low vs. High, p=0.477). Shaded areas represent 95% confidence intervals.

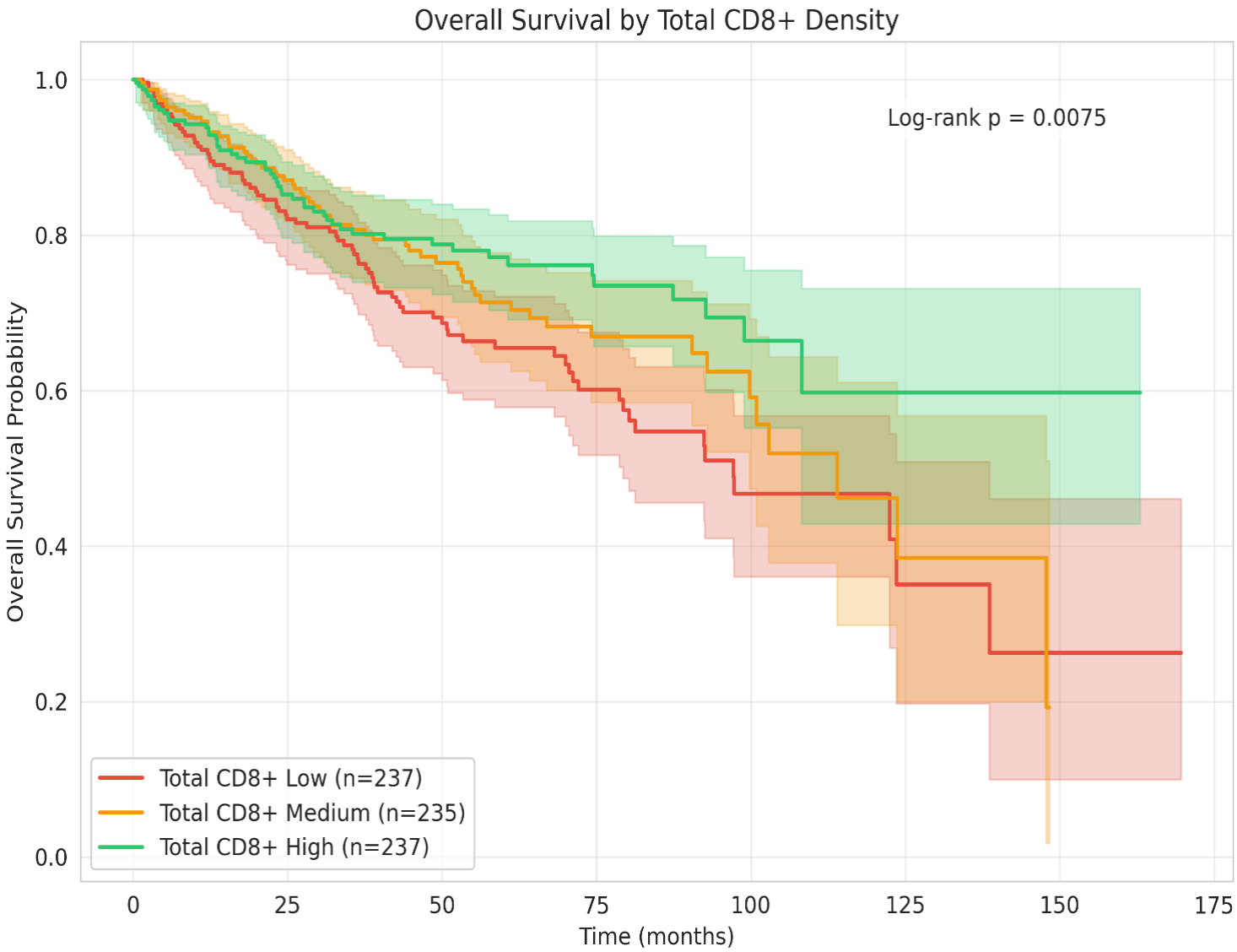


Figure 2

Kaplan-Meier Survival Analysis: Total CD8+ density. Patients were stratified into tertiles based on total CD8+ density (center + front): Low (≤344 cells/mm², n=237), Medium (345-724 cells/mm², n=235), and High (≥725 cells/mm², n=237). Higher total CD8+ density was associated with improved survival (log-rank: Low vs. High, p=0.008). Shaded areas represent 95% confidence intervals.

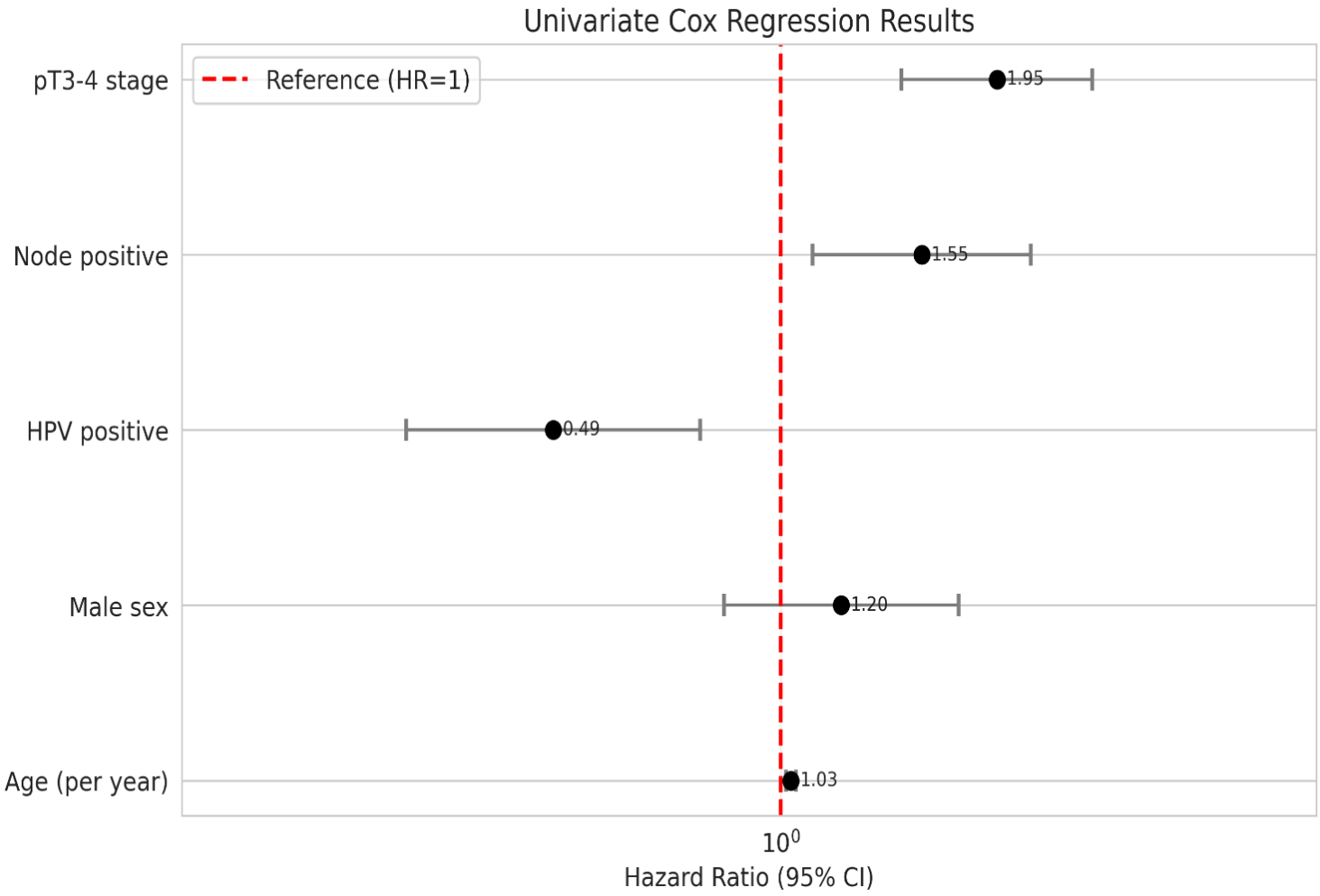


Figure 3

Forest Plot of Univariate Hazard Ratios. Forest plot showing hazard ratios (HR) and 95% confidence intervals from univariate Cox regression analysis. Established prognostic factors, age (HR=1.03 per year, p<0.001), HPV positivity (HR=0.49, p=0.006), nodal positivity (HR=1.55, p=0.002), and pT3-4 stage (HR=1.95, p<0.001), were independently associated with overall survival.