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Original Article

Tumor volume and cancer stem cell expression as prognostic markers for high-dose loco-regional failure in head and neck squamous cell carcinoma – A DAHANCA 19 study

Morten Horsholt Kristensen [a,*], Mia Kristina Sørensen [a,b], Trine Tramm [a,b,c], Jan Alsner [a], Brita Singers Sørensen [a,d], Christian Maare [e], Jørgen Johansen [f], Hanne Primdahl [g], Åse Bratland [h], Claus Andrup Kristensen [i], Maria Andersen [j], Jacob Kinggaard Lilja-Fischer [a,k], Anne Ivalu Sander Holm [g], Eva Samsøe [l], Christian Rønn Hansen [d,m,n], Ruta Zukauskaite [f], Jens Overgaard [a], Jesper Grau Eriksen [a]

[a] Department of Experimental Clinical Oncology, Aarhus University Hospital, Aarhus, Denmark
[b] Department of Pathology, Aarhus University Hospital, Aarhus, Denmark
[c] Department of Clinical Medicine, Aarhus University, Aarhus, Denmark
[d] Danish Centre for Particle Therapy, Aarhus University Hospital, Aarhus, Denmark
[e] Department of Oncology, Herlev Hospital, Herlev, Denmark
[f] Department of Oncology, Odense University Hospital, Odense, Denmark
[g] Department of Oncology, Aarhus University Hospital, Aarhus, Denmark
[h] Department of Oncology, Oslo University Hospital, Oslo, Norway
[i] Department of Oncology, Rigshospitalet, Copenhagen, Denmark
[j] Department of Oncology, Aalborg University Hospital, Aalborg, Denmark
[k] Department of Otolaryngology – Head & Neck Surgery, Aarhus University Hospital, Denmark
[l] Zealand University Hospital, Department of Oncology, Næstved, Denmark
[m] Odense University Hospital, Laboratory of Radiation Physics, Odense, Denmark
[n] University of Southern Denmark, Department of Clinical Research, Odense, Denmark

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ABSTRACT

Background and purpose: Reliable and accessible biomarkers for patients with Head and Neck Squamous Cell Carcinoma (HNSCC) are warranted for biologically driven radiotherapy (RT). This study aimed to investigate the prognostic value of putative cancer stem cell (CSC) markers, hypoxia, and tumor volume using loco-regional high-dose failure (HDF) as endpoint.

Materials and methods: Tumor tissue was retrieved from patients treated with primary chemo-(C-)RT and nimorazole for HNSCC in the Danish Head and Neck Cancer Study Group (DAHANCA) 19 study. Tumor volume, hypoxic classification, and expression of CSC markers CD44, *SLC3A2*, and *MET* were analyzed. For patients with eligible data on all parameters (n = 340), the risk of HDF following primary chemo-(C-)RT were analyzed by these biomarkers as a whole and stratified for p16-positive oropharynx (p16 + OPSCC) vs p16-negative (p16-) tumors (oral cavity, p16- oropharynx, hypopharynx and larynx).

Results: Higher risk of HDF was seen for patients with larger primary and nodal volume (>25 cm^3, Hazard Ratio (HR): 3.00 [95 % CI: 1.73–5.18]), high SLC3A2 (HR: 2.99 [1.28–6.99]), CD44 (>30 % positive, HR: 2.29 [1.05–5.00]), and p16- tumors (HR: 2.53 [1.05–6.11]). p16- tumors had a higher CSC marker expression than p16 + OPSCC. The factors associated with the highest risk of HDF were larger volume (HR: 3.29 [1.79–6.04]) for p16- tumors (n = 178) and high *SLC3A2* (HR: 6.19 [1.58–24.23]) for p16 + OPSCC (n = 162).

Conclusion: Tumor volume, p16, and CSC markers are potential biomarkers for HDF for patients with HNSCC treated with (C-)RT. Lower expression of CSC in p16 + OPSCC may contribute to better tumor control.

* Corresponding author.
E-mail address: mortenhorsholt@oncology.au.dk (M.H. Kristensen).

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Introduction

Following primary radiotherapy (RT), the outcome for patients with head and neck squamous cell carcinoma (HNSCC) has improved over the last decades [1]. This may partly be explained by the increasing frequency of human papillomavirus (HPV)-related oropharyngeal squamous cell carcinoma (OPSCC) [2], and partly by intensified treatment regimens including technical and biological optimization. In Denmark, loco-regional control has improved with the addition of hypoxic sensitization (nimorazole) [3], reduced overall treatment time (6 fractions/week), and concomitant chemotherapy, adjusted for the influence of stage and HPV-status [1]. Despite this, HNSCC remains a therapeutic challenge [4]. Dose-escalated hyperfractionated accelerated radiotherapy (HART) has been shown to improve local and regional control compared to conventional or altered fractionation schedules [5,6] and the addition of chemotherapy to HART showed superior overall survival compared to other treatment modalities [7]. However, as current treatment regimens are sufficient to achieve loco-regional control for the majority of patients, feasible biomarkers are warranted for identifying patients who may benefit from intensified treatment strategies.

Since most failures occur in the high-dose volume [8], radioresistance is the most likely reason for treatment failure for the majority of patients. Therefore, the focus remains on methods to stratify and potentially counteract differences in radiosensitivity. To achieve tumor control, all cancer stem cells (CSC) that hold the potential to differentiate into viable tumor cells need to be eradicated during primary RT. Thus, the number of CSCs is a crucial determinant for local control probability [9,10]. The number of CSCs can indirectly be perceived as a combination of tumor volume and density of CSCs [9–11].

The level of CSCs can be characterized by molecular markers for stemness, which are identified by in vivo-transplantation assays for the specific marker [12]. Three particular biomarkers that were assessed by transplantation assays to have stem-like capabilities are *SLC3A2* (gene coding for the heavy chain of CD98 (CD98hc))[13], *MET* (mesenchymal-epithelial transition gene) [14,15], and CD44 (cell surface glycoprotein) [16]. Linge et al. identified inferior loco-regional control for patients with large primary tumor volumes and total tumor volume (including both primary and nodal), high *SLC3A2* expression, and CD44 after primary (chemo-)radiotherapy (C-)RT [17] and for *MET* in a cohort treated with postoperative (C-)RT [18]. The influence of tumor volume, CD44, and *SLC3A2* was independently validated for overall survival, and CD44 protein maintained importance for loco-regional control [19].

Primary gross tumor volume (GTV-T) has been identified as a prognostic factor for local failure [20,21]. While this has been identified for local control in both p16-positive (p16 +) and p16-negative (p16-) OPSCC [22], other studies have found diverging results in oropharyngeal [23,24] or laryngeal [25] squamous cell carcinoma.

The aim of the present study was to identify patients with a higher risk of high-dose failure with the hypothesis that p16 (p16 + OPSCC vs p16- tumors), the expression of CSC markers, tumor volume, and hypoxia can identify more radioresistant tumors that tend to fail in the high-dose volume.

Materials and methods

This cohort study was based on the multicenter, phase III, randomized controlled trial DAHANCA (The Danish Head and Neck Cancer Study Group) 19 conducted from 2007 to 2012 [26]. Patients with HNSCC were treated with standard primary (C-)RT and nimorazole. Low-dose weekly cisplatin was given to patients with stage III-IV disease. Patients were randomized to either placebo or the EGFR-inhibitor zalutumumab. The addition of zalutumumab showed no effect on the primary endpoint of 5-year loco-regional control. Patient and tumor characteristics, and clinical outcome data, were available from the clinical DAHANCA database [27] with full GCP monitoring up to five years. DAHANCA 19 was conducted as an intention to treat-study, however, patients for the current study were only included if they completed curative primary (C-)RT. Flow diagram of the conducted biomarker analyses are presented in Fig. 1.

Tumor volume

The planning CT scans were collected in DcmCollab system [28] and primary tumor (GTV-T) and nodal (GTV-N) volumes measured in cm^3 were reviewed and extracted. If a tumor was confined to the palatine tonsil and the patient had a tonsillectomy as part of the diagnostic work-up, the tonsillar bed was considered as GTV-T. These patients were excluded from the volumetric analyses (Fig. 1).

Tumor tissue analyses

Formalin-fixed paraffin-embedded (FFPE), pre-treatment primary tumor tissue biopsies were collected and slides stained with hematoxylin and eosin (H&E). Two pathologists (MKS, TT) evaluated slides from each block to ensure that the FFPE block contained a sufficient amount of invasive tumor tissue (≥5 % of the tissue on the slide) (Fig. 1).

To evaluate hypoxia and the relative expression of the CSC markers *SLC3A2* and *MET*, sections of 7 μm of selected FFPE tissue were cut, from which RNA was extracted (Siemens Tissue Preparation System). On the extracted RNA, quantitative real-time polymerase chain reaction (qPCR) was performed using TaqMan gene expression assays (Life Technologies) [29,30]. The 15-gene hypoxia classifier developed by Toustrup et al. [31] was used to classify tumors as "more" or "less" hypoxic. The CSC markers, *SLC3A2* and *MET*, were included in the qPCR analysis, (TaqMan Gene Expression Assays: *C-MET*: Hs01565584_m1, *SLC3A2*: Hs00374243_m1, ThermoFisher Scientific), and the expression levels were analyzed by normalization to three pH- and hypoxia-independent reference genes (*RPL37A*, *ACTR3*, and *NDFIP1*). ΔCt values represented the specific gene's upregulation relative to the reference genes. These values were log-2 transformed prior to further analysis, where the log2-transformed value can be interpreted as the gene expression relative to the reference genes.

CD44 protein positivity was evaluated with immunohistochemistry. CD44 was evaluated at protein level instead of at gene level as CD44 is expressed in many polymorphisms and variants [32]. Three μm FFPE slices were cut and deparaffinized by heating (72 °C). Heat-induced epitope retrieval (97 °C at Ph = 9 for 4 min.) was applied before monoclonal antibody (clone 156-3C11, mouse, 1:400, ImmunoLogic, NL) incubation (16 min.) and hematoxylin incubation (8 min.). Negative and positive external control tissue (liver, pancreas, tonsil, and appendix) were included on each individual slide. Samples were scored by a pathologist (MKS) blinded to patient characteristics and outcomes. In cases of doubt, these were discussed with a senior pathologist (TT) until consensus. CD44-protein was semi-quantitatively determined as the fraction of CD44 tumor cells with positive membrane staining of all tumor cells in the sample. Examples are shown in Supplementary S1.

HPV/p16 status was evaluated with standard immunohistochemistry (clone JC8) using a cutoff of 70 % positivity in both nuclei and cytoplasm of the tumor cells [33]. In the present study, p16 was used to separate patients into two groups of HNSCC: p16 + OPSCC (p16-positive oropharyngeal) and p16- tumors (p16- oropharyngeal and oral cavity hypopharyngeal and laryngeal irrespective of p16-status).

For all analyses, only patients with eligible data on all parameters (CD44, tumor volume, *SLC3A2*, *MET*, and hypoxia) were included (n = 340; Suppl. S2).

Statistics

Hazard ratio (HR) plots were made for each variable to identify the optimal cutoff point for the variables (volume and CSCs; method described in Suppl. S3) and resulted in cutoff values of 30 % (30 % included in low-value group) for CD44, 25 cm^3 for GTV-tot, −4.40

(log2) for *SLC3A2*, and −4.53 (log2) for *MET* (Suppl. S3).

The endpoint high-dose failure was used for all analyses, determined by analysis of the radiation dose to the estimated point of origin of the failure as described in [8] (Suppl. S4). In time-to-event analyses, the endpoint was the first high-dose failure calculated from the day of the last fraction. The cumulative incidence of high-dose T-, N- or T + N-site failure within five years was analyzed with the Aalen-Johansen estimator. Competing risk were distant failure, death from any cause, and loco-regional failure with missing scans or non-high-dose failure. Hazard ratios were estimated using cause-specific Cox-regression. The potential biomarkers were included based on the identified cutoff points (Suppl. S3) and as continuous parameters for GTV-T and GTV-N, respectively.

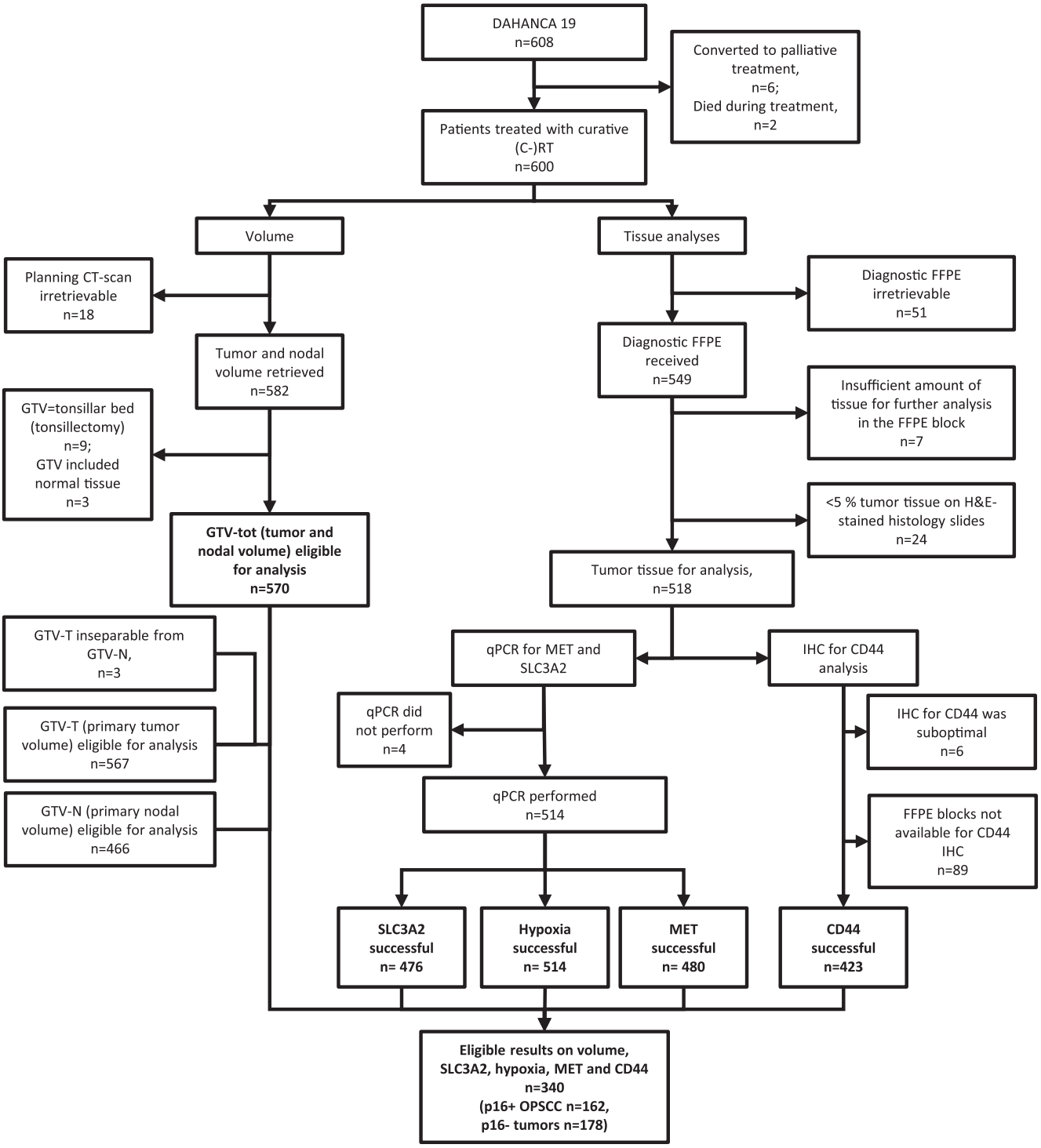

Fig. 1. Flow diagram of volume and tumor tissue analyses of all patients in DAHANCA 19; bold indicates patients with successful analysis for the specific parameter. CTV1: high-dose volume; (C-)RT: (Chemo-)Radiotherapy; GTV: gross tumor volume; GTV-T gross tumor volume for the primary tumor; GTV-N gross tumor volume for nodal disease; FFPE: formalin-fixed paraffin-embedded; H&E: hematoxylin and eosin; qPCR: quantitative real-time polymerase chain reaction; IHC: immuno-histochemistry; p16 + OPSCC: patients with HPV/p16-positive oropharyngeal squamous cell carcinoma; p16- tumors: oral cavity, HPV/p16-negative oropharyngeal, hypopharyngeal and laryngeal squamous cell carcinoma.

Ethics

Approval (Clinical Trials: NCT00496652) was granted by The Central Denmark Region Committees on Health Research Ethics (65532) and The Southern Norwegian Research Ethics Committee (S-073774b).

Results

A total of 340 patients with eligible results on all four parameters (Fig. 1, Table 1 and Suppl. S2) were included in the time-to-event analyses. The included group contained relatively more patients in WHO Performance Status 1 and 2, more treated with HART (6 % vs 1 %), and more receiving no concomitant chemotherapy (33 % vs 22 %) compared to the patients with one or more missing values (Table 1). The expression of *SLC3A2* was higher (median: −4.13 vs −3.92, $p = .001$) for the excluded patients, and *MET* was lower (median −4.35 vs −4.13, $p = .004$), whereas volumes, CD44 expression and hypoxia classification showed higher homogeneity between the groups.

For the 340 included patients, 83 loco-regional failures were diagnosed within 5 years. Point of origin analysis was successful for 75 patients, whereas no failure or planning CT scans were available for 8 patients. Of 75 patients, 64 had a high-dose volume failure (T-site: n = 34; N-site: n = 20; T + N: n = 10), and 11 patients had failure sites not covered by 95 % of the prescribed high dose.

In univariable analyses, all variables were prognosticators for high-dose failure based on the cutoffs (Fig. 2). Higher risk for high-dose failure was identified for patients with p16- tumors (HR: 3.94 [95 % CI: 2.18–7.15]), for > 30 % tumor cells positive for CD44 (HR: 3.42 [1.86–6.29]), total tumor volume (GTV-T and GTV-N) larger than 25 cm^3 (HR: 2.17 [1.28–3.68]), tumors with *SLC3A2* expression higher than −4.40 (HR: 3.83 [1.95–7.52]), *MET* expression higher than −4.53 (HR: 3.13 [1.67–5.87]), and for patients with tumors classified as more hypoxic (HR: 1.91 [1.17–3.13]).

In the multivariable analyses, tumor volume was included both as a

Table 1
Baseline patient, tumor, and treatment characteristics, including volume, expression of stem cell markers, and hypoxic classification. Listed are patients included in the analysis with eligible data on all parameters (volume, CD44, SLC3A2, MET, hypoxia classification), where the not included group consists of patients with one or more missing values. Data are presented as *n* and (%) for categorical measures and median (IQR) for continuous. Tests for difference between groups were performed with Pearson's chi2 for categorical variables with ≥ 5 patients in all cells and Fischer's exact if a cell contained < 5 patients. PS: performance status; WHO: World Health Organization; PY: Pack years; Stage: IUCC 8th ed.; fx/w: fractions per week.

	All	Not included	Included	*p*
	n = 600	*n = 260*	*n = 340*	
Sex				
Male	492 (82 %)	215 (83 %)	277 (81 %)	*0.70*
Female	108 (18 %)	45 (17 %)	63 (19 %)	
Age	58 (53–64)	59 (53–64)	58 (53–64)	*0.44*
PS (WHO)				
0	459 (77 %)	218 (84 %)	241 (71 %)	*<0.001*
1	125 (21 %)	39 (15 %)	86 (25 %)	
2	16 (3 %)	3 (1 %)	13 (4 %)	
Smoking				
<30 PY	292 (49 %)	136 (52 %)	156 (46 %)	*0.12*
>=30 PY	308 (51 %)	124 (48 %)	184 (54 %)	
Tumor site				
p16 + OPSCC	306 (51 %)	144 (55 %)	162 (48 %)	*0.31*
p16-OPSCC	114 (19 %)	48 (18 %)	66 (19 %)	
Oral cavity	22 (4 %)	10 (4 %)	12 (4 %)	
Hypopharynx	71 (12 %)	27 (10 %)	44 (13 %)	
Larynx	87 (14 %)	31 (12 %)	56 (16 %)	
Stage (UICC8)				
I	65 (11 %)	31 (12 %)	34 (10 %)	*0.20*
II	232 (39 %)	90 (35 %)	142 (42 %)	
III	118 (20 %)	61 (23 %)	57 (17 %)	
IV A	172 (29 %)	73 (28 %)	99 (29 %)	
IV B	13 (2 %)	5 (2 %)	8 (2 %)	
Treatment				
66–68 Gy, 6fx/w	577 (96 %)	258 (99 %)	319 (94 %)	*<0.001*
76 Gy, 10fx/w	23 (4 %)	2 (1 %)	21 (6 %)	
No chemo	170 (28 %)	57 (22 %)	113 (33 %)	*0.002*
Chemo	430 (72 %)	203 (78 %)	227 (67 %)	
GTV-tot (cm^3)	27 (15–48)	25 (16–44)	27 (15–50)	*0.58*
GTV-T (cm^3)	13 (7–27)	14 (7–26)	13 (7–30)	*0.34*
GTV-N (cm^3)	11 (4–24)	10 (4–20)	12 (4–25)	*0.35*
Volume (GTV-tot)				
≤25 cm^3	270 (47 %)	114 (50 %)	156 (46 %)	*0.39*
>25 cm^3	300 (53 %)	116 (50 %)	184 (54 %)	
SLC3A2(log2)	-4.06 (-4.56–3.58)	-3.92 (-4.38–3.45)	-4.13 (-4.68–3.64)	*0.001*
≤-4.40	162 (34 %)	33 (24 %)	129 (38 %)	*0.004*
>-4.40	314 (66 %)	103 (76 %)	211 (62 %)	
MET(log2)	-4.21 (-5.12–3.35)	-4.35 (-5.36–3.52)	-4.13 (-5.01–3.29)	*0.043*
≤-4.45	192 (37 %)	61 (35 %)	131 (39 %)	*0.44*
>-4.45	322 (63 %)	113 (65 %)	209 (61 %)	
CD44 protein (%)	50 (10–80)	30 (5–70)	50 (10–80)	*0.17*
≤30 %	189 (45 %)	42 (51 %)	147 (43 %)	*0.23*
>30 %	234 (55 %)	41 (49 %)	193 (57 %)	
Hypoxia				
Less	297 (58 %)	101 (58 %)	196 (58 %)	*0.93*
More	217 (42 %)	73 (42 %)	144 (42 %)	

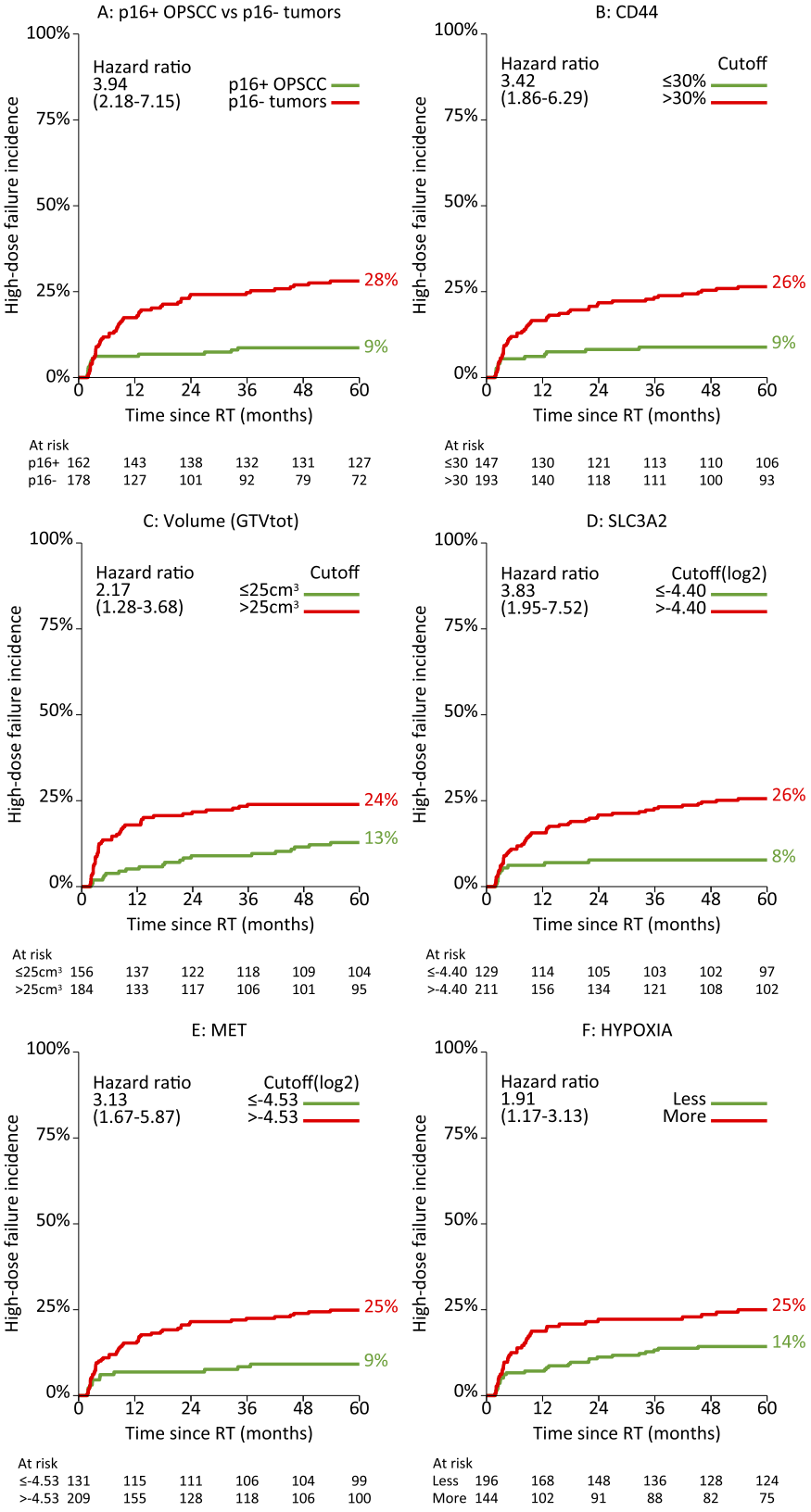

Fig. 2. Cumulative incidence of high-dose failure based on the cutoff point for each variable. The Aalen-Johansen versions of the Kaplan-Meier estimator curves show high-dose failure as the event and loco-regional failure with missing scans or non-high-dose failure, distant failure and death from any cause as competing risks. Time was calculated from the last day of (RT) until five years in months. Green curves show the low-value group, while red indicates the high value from the cutoff (Suppl. Figure S3). A: p16 + OPSCC vs p16- tumors; B: CD44 positivity; C): Volume (GTV-tot (GTV-T + GTV-N)); D): *SLC3A2*; E): *MET*; F): Hypoxic classification according to the 15-gene hypoxia classifier. Hazard ratios and (95 % CI) were calculated by cause-specific Cox regression. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

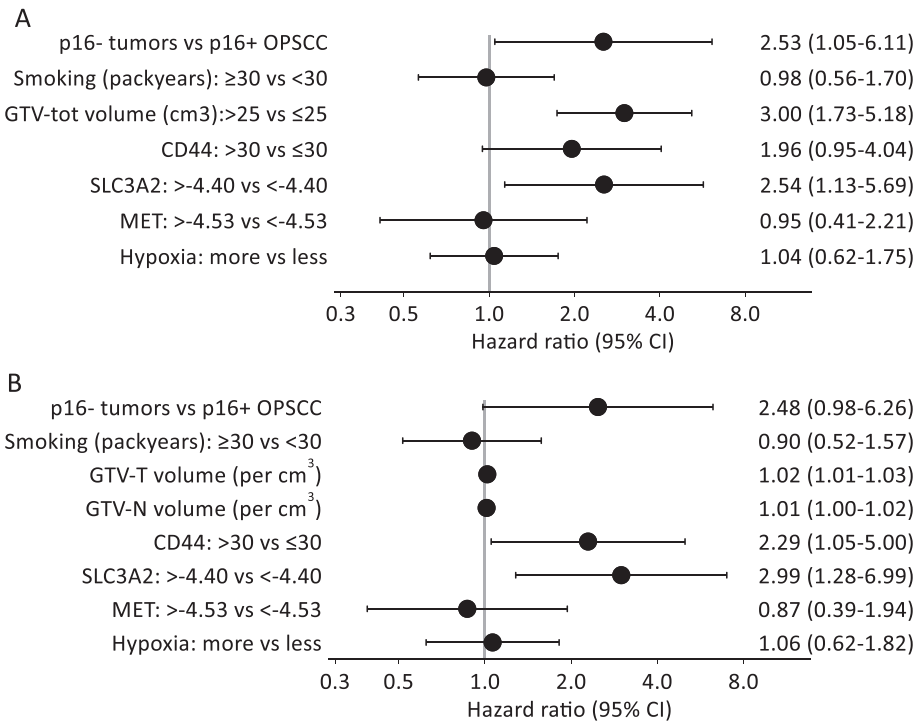

Fig. 3. Multivariable Cox regression of potential biomarkers. Five-year high-dose failure was the endpoint. A) Volume as a dichotomous variable based on the identified cutoff and B) as continuous variables for both GTV-T and GTV-N. GTV-N was converted to 0 cm^3 instead of missing for patients with N0-disease. X-axis (hazard ratio and 95 % CI) is log-scaled. For *SLC3A2*, *MET*, CD44, and volume (in A), the high and low-value groups were based on the cutoff value from Suppl. Figure S3.

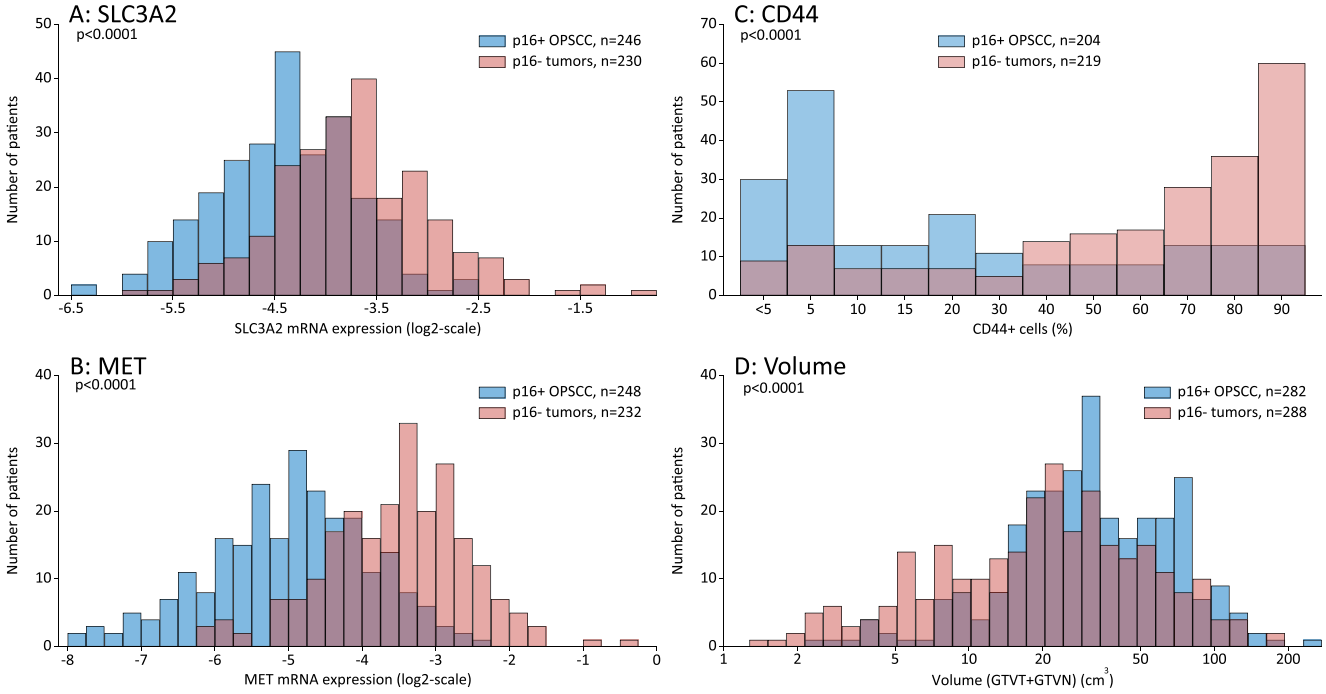

Fig. 4. Histograms of the frequency for the four variables by the number of patients: A) Expression of *SLC3A2* mRNA relative to the reference genes (log2); B) Expression of *MET* mRNA relative to the reference genes (log2); C) The fraction of CD44 positive tumor cells among all tumor cells on the immunohistochemistry slide (% positive); D) Accumulated volume (GTV-tot) of the primary tumor (GTV-T) and regional lymph nodes (GTV-N) (cm^3); y-axis: Number of patients; the bars are created with 50 % opacity, so the purple color indicates the overlap; blue: Patients with p16-positive oropharyngeal squamous cell carcinoma (p16 + OPSCC); red: patients with oral cavity, p16-negative oropharyngeal, hypopharyngeal, and laryngeal squamous cell carcinoma (p16- tumors). Test for difference between groups: A and B: *t*-test; C and D: log-rank. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

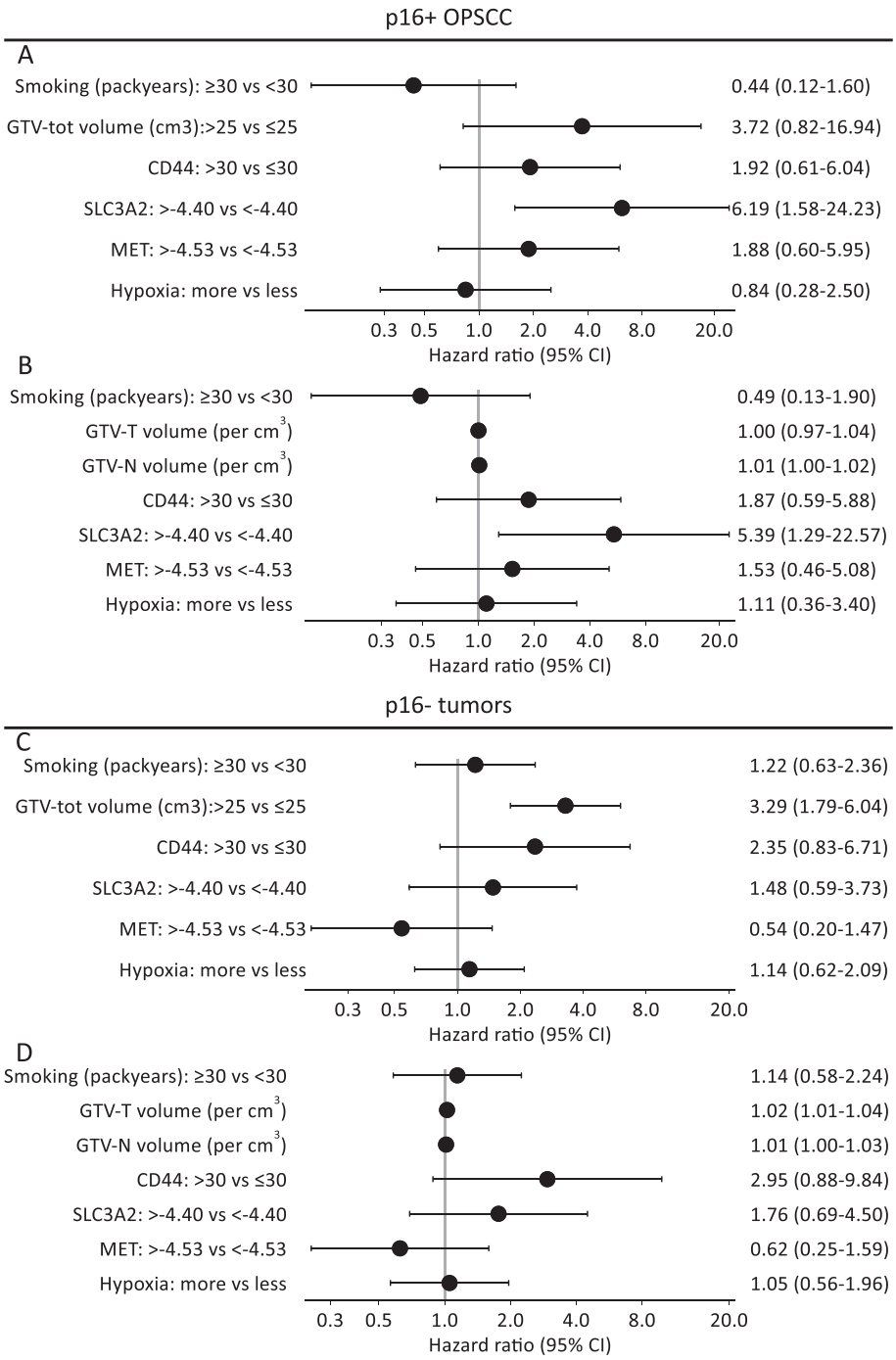

Fig. 5. Multivariable Cox-regression with 5-year high-dose failure as the endpoint for CSC markers, hypoxia, and volume for p16 + OPSCC in A) and B) and p16- tumors in C) and D). In A) and C): Volume included as a grouping variable based on the identified cutoff. In B) and D), volume included as continuous variables for both GTV-T and GTV-N. X-axis (hazard ratio and 95 % CI) is log-scaled.

dichotomized variable (GTV-tot $\geq$ 25 or $<$ 25 cm^3) and separated as continuous variables with GTV-T and GTV-N reported in cm^3. Including volume as a dichotomous variable (Fig. 3A), higher risk of high-dose failure was found for patients with p16- tumors (HR: 2.53 [1.05–6.11]), larger volume (HR: 3.00 [1.73–5.18]), and high SLC3A2-expression (HR: 2.54 [1.13–5.69]). High CD44-expression showed a strong trend for higher risk of high-dose failure (HR: 1.96 [0.95–4.04]), while smoking, MET, and hypoxia classification showed no prognostic effect in the multivariable model. Including primary tumor (GTV-T) and nodal (GTV-N) as continuous variables (Fig. 3B) did not alter the overall results, except for reducing the prognostic impact of p16- tumors (HR: 2.48 (0.98–6.26)). Both GTV-T and GTV-N showed independent prognostic effect with an increased risk of high-dose failure of 2 % (95 % CI: 1–3 %) and 1 % (95 % CI: 0.4–2.2 %) per increase in cm^3 for GTV-T and GTV-N, respectively. The multivariate analyses including log2-transformed volumes showed similar results (Suppl. S5A).

As p16 + OPSCC and p16- tumors can be regarded as two distinct entities, both with respect to the biology [34] and prognosis [35], the possible differences in the expressions of CSC markers and collective tumor volume between the two groups were explored for all eligible values. Patients with p16- tumors had higher CSC marker expressions and smaller combined primary and nodal volumes compared to p16 +

OPSCC (Fig. 4). For the 162 patients with p16 + OPSCC, higher risk of high-dose failure was observed for patients with higher *SLC3A2* and *MET* expression, while a trend was observed for larger volume (Suppl. S6). In multivariable analysis (Fig. 5A-B, Suppl. S5B), SLC3A2 was the only parameter with an impact on the risk of high-dose failure for p16 + OPSCC (HR: 6.19 [1.58–24.23]) (Fig. 5A). The effect of tumor volume as a dichotomous variable (HR: 3.72 [0.82–16.94]), CD44 (HR: 1.92 [0.61–6.04]), and *MET* (HR: 1.88 [0.60–5.95]) showed a trend for increased risk for the high-value groups.

For the 178 patients with p16- tumors, univariable analysis revealed collective dichotomized GTV-tot as the variable with the most pronounced prognostic value (HR: 3.18 [1.78–5.68]) (Suppl. S7). A trend was observed for higher risk of high-dose failure for patients with higher CD44 (HR: 2.72 [0.98–7.57]). In a model including all factors, larger volumes were associated with an increased risk of high-dose failure (Fig. 5C-D, Suppl. S5C). Both dichotomous GTV-tot (Fig. 5C) but also continuous GTV-T volume showed a 2 % [1–4 %] increased risk per cm^3 increase in GTV-T (Fig. 5D).

Hypoxia showed no prognostic value in the multivariable analyses (Figs. 3 and 5). To evaluate possible interactions between the included biomarkers, correlation analyses was performed. A relationship between *SLC3A2* and MET (R = 0.54, Pearson's correlation) was observed. All CSC markers and p16-status, but not volume, showed a weak correlation to hypoxia (Suppl. S8).

Discussion

The study demonstrated that p16-negativity, higher expression of the CSC markers *SLC3A2* and CD44, and larger tumor volume were associated with increased risk of high-dose failure. Hypoxia evaluated by the 15-gene classifier showed prognostic effect only in univariate analyses. These findings align with results from the German Cancer Consortium Radiation Oncology Group (DKTK-ROG) [17], in a cohort of patients treated with primary RT. In that study, CD44, *SLC3A2*, p16- tumors, and total tumor volume were associated with worse loco-regional control within the irradiated volume. Additionally, the DKTK-study identified a negative prognostic effect of more hypoxic tumors for patients with smaller ($\leq$19 cm^3) tumor volume in univariate analyses also using the 15-gene classifier. Despite differences in the patient cohorts and methods for analyzing the biomarkers between the present study and the DKTK-study [17], similar conclusions were reached: p16 status, tumor volume, and CSC markers are important factors for failure in the irradiated volume/high-dose failure.

In the current study, the biological profile differed between patients with p16 + OPSCC and p16- tumors, where higher expressions of CD44, *SLC3A2*, and *MET* were observed in patients with p16- tumors. Linge et al [17] also identified lower CSC markers CD44 and *SLC3A2* for patients with p16 + tumors. Lower expression of CSCs in p16 + OPSCC might support the overall better outcomes observed for this patient group.

High *SLC3A2*-expression was associated with increased risk of high-dose failure, especially for p16 + OPSCC (Fig. 5 and Suppl. S6). Similarly, a negative prognostic value of CD98 (encoded by *SLC3A2*) only in p16 + OPSCC was found by Rietbergen et al. [36].

The role of CD44 as a prognostic marker for outcome is debatable. In line with previous results, CD44 positivity was, in this study, linked to HPV status; patients with p16 + tumors showed lower expression [17,36] (Fig. 4C and Suppl. S8). Without stratification for HPV status, higher CD44 was linked to worse outcome (Fig. 3B), in accordance with other studies [17,37,38]. Stratification based on p16 + OPSCC and p16- tumors is essential in biomarker-based evaluation of the effect of CD44 on the risk of failure since the impact of CD44 otherwise might reflect the p16-positivity in OPSCC.

Patel et al. found CD44 to have no significant impact at any cutoff for p16- tumors [39], whereas, in a similar study, higher CD44 correlated with worse loco-regional control [40]. Both studies identified markers for hypoxic tumors as prognostic factors for worse outcome, which highlights a difference in the present cohort compared to others since DAHANCA guidelines recommend concomitant hypoxic radiosensitization. Hypoxia is a significant determinant of loco-regional control [17,31,40]. The response to nimorazole may be predicted by biomarkers [41]. However, in the current study, hypoxia classification did not show prognostic effect in the multivariable models (Figs. 3 and 5). *MET*, *SLC3A2*, and CD44 had only weak correlation to hypoxic classification (Suppl. S8), which aligns with independent cohorts [17,18,40]. Furthermore, hypoxia upregulates CSC via hypoxia-inducible factor (*HIF1α*) and epidermal to mesenchymal transition [42]. Supposedly, hypoxic niches protect against radiation-induced oxidative stress or induce a slow cell cycling state of CSCs [36]. Likely, a direct or indirect interaction between hypoxia and CSCs exists. In that case, the effect on outcome of the CSC biomarkers in this study might be dampened by intervention against hypoxia (nimorazole).

Large primary tumor volume has been determined to be an independent poor prognostic factor for local control [43–45], also when stratified for HPV/p16 status [22]. Similar results was found regarding loco-regional control, including combined primary and nodal volume [44]. Both dichotomized total volume (Fig. 3A) and volume as continuous parameters for T- and N- site (Fig. 3B and S5) had prognostic value for high-dose failure. Larger lymph nodes is often observed in p16 + OPSCC, often with a cystic central component. Despite this, the present study showed that nodal volume for p16 + OPSCC was associated with increased risk of high-dose failure (Fig. 5B and S5B). However, the tumor volume effect was most pronounced for p16- tumors and seemed primarily related to primary (T-site) volume. By the stem-cell model, there might be a linear correlation between volume and the number of CSCs [9]. Should CD44, *MET*, and *SLC3A2* be actual markers of the density of CSC, the density factor might be less important than volume in p16- tumors. This may be due to differences in the tumor microenvironment (i.e., loss of hierarchy with increased volume, oxygen supply, and immune cells) that might influence the stem-like potential of cells. Thus, volume might be the best surrogate marker for the number of CSCs in p16- tumors.

A limitation of the biological analyses (CSCs and hypoxia) in the present study, is the assumption of tumor and nodal homogeneity. Biological analyses were performed on diagnostic tumor tissue biopsies from T-site. If there is a difference in the number of CSCs between more or less active spatial tumor microenvironments is unknown. Another limitation is that the cutoffs in the current series introduce a bias since they were chosen based on criteria from hazard-ratio plots in contrast to methods where a cutoff is produced automatically by an entirely data-driven method. However, the model in the present study was based on objective criteria (Suppl. S3). This approach was an a priori choice by the authors to enhance visibility and reproducibility. Nonetheless, these results need to be validated in an independent cohort, since this was a hypothesis generating study.

In conclusion, the results from this study support the hypothesis that p16 (p16 + OPSCC vs p16- tumors), expression of cancer stem cell markers *SLC3A2* and CD44, and tumor volume are prognostic biomarkers for high-dose failure after primary (C-)RT in HNSCC, in line with previous studies [17,19]. Hypoxia was not associated with increased risk of high-dose failure, probably because patients received hypoxic radiosensitization with nimorazole. For p16 + OPSCC, a lower expression of CSCs was observed, which adds to the potential explanation for the overall superior outcomes for these patients. For patients with p16 + OPSCC, *SLC3A2* was the most significant marker for high-dose failure, whereas volume was the main factor for p16- tumors.

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CRediT authorship contribution statement

Morten Horsholt Kristensen: Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Mia Kristina Sørensen:** Writing – review & editing, Visualization, Validation, Resources, Methodology, Formal analysis, Data curation. **Trine Tramm:** Writing – review & editing, Visualization, Supervision, Methodology, Formal analysis, Data curation. **Jan Alsner:** Writing – review & editing, Visualization, Software, Methodology, Formal analysis. **Brita Singers Sørensen:** Writing – review & editing, Validation, Project administration, Methodology. **Christian Maare:** Writing – review & editing, Investigation, Data curation. **Jørgen Johansen:** Writing – review & editing, Investigation, Data curation. **Hanne Primdahl:** Writing – review & editing, Investigation. **Åse Bratland:** Writing – review & editing, Investigation, Data curation. **Claus Andrup Kristensen:** Writing – review & editing, Investigation. **Maria Andersen:** Writing – review & editing, Investigation. **Jacob Kinggaard Lilja-Fischer:** Writing – review & editing, Investigation. **Anne Ivalu Sander Holm:** Writing – review & editing, Investigation. **Eva Samsøe:** Writing – review & editing, Investigation, Data curation. **Christian Rønn Hansen:** Writing – review & editing, Investigation, Data curation. **Ruta Zukauskaite:** Writing – review & editing, Methodology, Investigation, Data curation. **Jens Overgaard:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Jesper Grau Eriksen:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.radonc.2024.110149.

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