



The value of plasma hypoxia markers for predicting imaging-based hypoxia in patients with head-and-neck cancers undergoing definitive chemoradiation

Alexander Rühle^{a,b}, Anca-L. Grosu^{a,b}, Nicole Wiedenmann^{a,b}, Juri Ruf^c, Birgit Bieber^{a,b}, Raluca Stoian^{a,b}, Andreas R. Thomsen^{a,b}, Eleni Gkika^{a,b}, Peter Vaupel^{a,b}, Dimos Baltas^{a,b}, Wolfgang A. Weber^d, Michael Mix^c, Nils H. Nicolay^{a,b,*}

^a Department of Radiation Oncology, Medical Center – University of Freiburg, Faculty of Medicine, University of Freiburg, Freiburg, Germany

^b German Cancer Consortium (DKTK), Partner Site Freiburg, German Cancer Research Center (DKFZ), Heidelberg, Germany

^c Department of Nuclear Medicine, Medical Center – University of Freiburg, Faculty of Medicine, University of Freiburg, Freiburg, Germany

^d Department of Nuclear Medicine, Technical University of Munich, Munich, Germany

ARTICLE INFO

Keywords:

Head-and-neck cancer
Radiotherapy
Osteopontin
FMISO-PET
Biomarker

ABSTRACT

Background: Tumor hypoxia worsens the prognosis of head-and-neck squamous cell carcinoma (HNSCC) patients, and plasma hypoxia markers may be used as biomarkers for radiotherapy personalization. We therefore investigated the role of the hypoxia-associated plasma proteins osteopontin, galectin-3, vascular endothelial growth factor (VEGF) and connective tissue growth factor (CTGF) as surrogate markers for imaging-based tumor hypoxia.

Methods: Serial blood samples of HNSCC patients receiving chemoradiation within a prospective trial were analyzed for osteopontin, galectin-3, VEGF and CTGF concentrations. Tumor hypoxia was quantified in treatment weeks 0, 2 and 5 using [¹⁸F]FMISO PET/CT. The association between PET-defined hypoxia and the plasma markers was determined using Pearson's correlation analyses. Receiver-operating characteristic analyses were conducted to reveal the diagnostic value of the hypoxia markers.

Results: Baseline osteopontin ($r = 0.579$, $p < 0.01$) and galectin-3 ($r = 0.429$, $p < 0.05$) correlated with the hypoxic subvolume (HSV) prior to radiotherapy, whereas VEGF ($r = 0.196$, $p = 0.36$) and CTGF ($r = 0.314$, $p = 0.12$) showed no association. Patients with an HSV > 1 mL in week 2 exhibited increased VEGF ($p < 0.05$) and CTGF ($p < 0.05$) levels in week 5. Pretherapeutic osteopontin levels were higher in patients exhibiting residual hypoxia at the end of treatment (104.7 vs. 60.8 ng/mL, $p < 0.05$) and could therefore predict residual hypoxia (AUC = 0.821, 95% CI 0.604–1.000, $p < 0.05$).

Conclusion: In this exploratory analysis, osteopontin correlated with the initial HSV and with residual tumor hypoxia; therefore, there may be a rationale to study hypoxic modification based on osteopontin levels. However, as plasma hypoxia markers do not correspond to any spatial information of tumor hypoxia, they have limitations regarding the replacement of [¹⁸F]FMISO PET-based focal treatments. The results need to be validated in larger patient cohorts to draw definitive conclusions.

Introduction

Despite improvements in diagnostics and treatment, head-and-neck squamous cell carcinoma (HNSCC) still is a major cause for cancer-related morbidity and mortality [1,2]. Radiotherapy, either alone or with concomitant chemotherapy, constitutes a main treatment modality for HNSCC [3–7]. The negative prognostic role of tumor hypoxia for the

outcome of HNSCC patients undergoing chemoradiation is well established [8–12], and several strategies have been chosen to specifically target tumor hypoxia, e.g., carbogen plus nicotinamide, hyperbaric oxygen, nitroimidazole and hyperthermia [13–18]. Radiotherapy dose escalation or usage of high linear energy transfer (LET) irradiation are other potential strategies used to overcome hypoxia-induced radioresistance [16,17,19]. On the other hand, human papillomavirus (HPV)-

* Corresponding author at: Medical Center – University of Freiburg, Department of Radiation Oncology, Robert-Koch-Str. 3, Freiburg 79106, Germany.

E-mail address: nils.nicolay@uniklinik-freiburg.de (N.H. Nicolay).

<https://doi.org/10.1016/j.ctro.2022.02.008>

Received 16 December 2021; Received in revised form 15 February 2022; Accepted 17 February 2022

Available online 21 February 2022

2405-6308/© 2022 Published by Elsevier B.V. on behalf of European Society for Radiotherapy and Oncology. This is an open access article under the CC

BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

positive HNSCC patients with absent or early resolving tumor-associated hypoxia may be candidates for treatment de-escalation due to their excellent prognosis [20,21].

Fluorine-18 misonidazole positron emission tomography (^{18}F]FMISO PET) is a non-invasive imaging method, allowing for detection and quantification of spatial hypoxia distribution [22,23]. ^{18}F]FMISO PET (and hypoxia PET imaging with other tracers such as fluorine-18 azomycin arabinoside (^{18}F]FAZA)) can be considered as gold standard for hypoxia imaging [24,25]; however, ^{18}F]FMISO PET is available only in few high-volume tertiary treatment centers so far. Surrogates for tumor hypoxia such as blood markers, gene signatures or magnetic resonance imaging (MRI) features could therefore facilitate hypoxia-based radiotherapy personalization approaches in the future [13,26–29].

In this post-hoc analysis of a prospective trial including HNSCC patients undergoing definitive chemoradiation, we aimed to examine the role of the plasma hypoxia markers osteopontin, galectin-3, VEGF and CTGF role as potential surrogate measures for ^{18}F]FMISO PET-based tumor hypoxia. Although all parameters have been described to be up-regulated by hypoxia, the exact correlations of these markers with the ^{18}F]FMISO PET-based hypoxia volume are largely unknown [30–34].

Methods

Patient treatment

The ^{18}F]FMISO trial was registered in the German Clinical Trial Register (DRKS00003830) and was approved by the institutional ethical review committee of the University of Freiburg (reference no. 479/12) in advance. It was conducted compliant with the Declaration of Helsinki (revised version of 2008), and all patients provided written informed consent prior to enrolment.

Longitudinal blood samples were available for a total of 27 patients. Patient characteristics of this population are shown in [supplementary table 1 \(Table S1\)](#). Patients underwent definitive cisplatin-based chemoradiation with doses of 50–54 Gy (EQD2) to the low-risk planning target volume (PTV) and 70 Gy (EQD2), delivered either as sequential or simultaneous boost, to the high-risk PTV. Three cycles of high-dose cisplatin (100 mg/m² body surface area in weeks 1, 4 and 7) were administered simultaneously during radiotherapy.

Imaging

At baseline, patients received computed tomography (CT), fluorine-18-deoxyglucose (^{18}F]FDG) and ^{18}F]FMISO PET/CT imaging. ^{18}F]FMISO PET/CT imaging was repeated in weeks 2 and 5 of treatment. ^{18}F]FMISO PET/CT imaging was carried out in radiation treatment position utilizing a thermoplastic head immobilization mask at 150 min post intravenous injection of 3.7 MBq/kg ^{18}F]FMISO (maximum activity of 370 MBq).

Both ^{18}F]FMISO and ^{18}F]FDG PET/CT images (and MRI, if available) were co-registered with the corresponding planning using Eclipse™ software (Varian Medical Systems, Palo Alto, CA, USA). The gross tumor volumes (GTVs) for the primary tumor and metastatic lymph nodes were delineated on the planning CT by incorporating the information of the complementary imaging techniques. All voxels within the primary and nodal GTVs were considered as hypoxic if the ratio of ^{18}F]FMISO SUV to mean SUV in the contralateral sternocleidomastoid muscle (^{18}F]FMISO SUV tumor-to-muscle ratio) was 1.4 or above. The volumes of the hypoxic subvolumes (HSVs) within the primary and nodal GTVs were calculated prior to radiotherapy and in week 2 and 5 during chemoradiation. Delineation of primary tumors and metastatic lymph nodes as well as quantification of the HSV was conducted with Eclipse™ software. The imaging parameters of the ^{18}F]FMISO PET/CT were described previously [35]. Following a previous study, we performed two separate analyses with different cut-off values

(either 0 mL or 1 mL) for the HSV regarding hypoxia resolution in week 2 [36]. As only two patients had an HSV >1 mL in week 5, not enabling reasonable statistical analyses, we used 0 mL as solitary cut-off value for week 5.

Blood sampling and analyses

Blood sampling was conducted at the same times of the corresponding ^{18}F]FMISO PET/CT scans. Blood was taken in week 0, 2 and 5 of chemoradiation and collected in EDTA monovettes® (Sarstedt, Nümbrecht, Germany). Blood samples were cooled on ice and centrifuged at 500g for 10 min at 4 °C. Subsequently, the supernatant was aliquoted into Nalgene™ Cryogenic storage tubes (Nalgene® Labware, Rochester, NY, USA) and stored at –80 °C. Enzyme-linked immunosorbent assays (ELISAs) for osteopontin (Human Osteopontin Quantikine ELISA Kit, DOST00, R&D Systems, Minneapolis, MN, USA), galectin-3 (Human Galectin-3 Quantikine ELISA Kit, DGAL30, R&D Systems), VEGF (Human VEGF Quantikine ELISA Kit, DVE00, R&D Systems) and CTGF (Human CTGF ELISA Kit, ab261851, Abcam, Cambridge, UK) were carried out based on the manufacturers' instructions.

Statistical analyses

Osteopontin, galectin-3, VEGF and CTGF plasma concentration as well as the HSVs were presented as mean values with standard deviations. The dynamics of plasma hypoxia markers during chemoradiation were examined with mixed model analyses and post-hoc Tukey tests. Correlations between plasma hypoxia markers and HSV were investigated using Pearson's correlations. A chi-square test was carried out to reveal whether the HPV status influenced early hypoxia resolution in our cohort. A multiple linear regression analysis with osteopontin plasma concentration in week 0 as a dependent variable was performed. Receiver-operating characteristic (ROC) analyses were conducted to determine the diagnostic power of osteopontin levels for residual hypoxia prediction. Both sensitivity and specificity of different osteopontin cutoffs regarding the prediction of residual hypoxia in week 5 were assessed. Cox regression analyses for locoregional control (LRC), progression-free survival (PFS) and overall survival (OS) were performed for the different plasma hypoxia markers, and hazard ratios (HR) with the corresponding 95% confidence intervals (95% CI) were indicated. $P \leq 0.05$ was considered statistically significant throughout the study. Both SPSS Statistics software version 25 (IBM, Armonk, NY, USA) and GraphPad version 8.2.1 (GraphPad Software, San Diego, CA, USA) were used for statistical analyses.

Results

Tumor hypoxia and hypoxia biomarkers show dynamic patterns during treatment

Total tumoral HSV (HSV within the primary GTV and the nodal GTVs) ranged at 13.6 ± 18.9 mL (mean \pm standard deviation), 6.0 ± 8.6 mL and 0.3 ± 0.6 mL in weeks 0, 2 and 5 of chemoradiation, respectively, displaying a significant decrease of tumor hypoxia between week 0 and 5 ($p < 0.01$, mixed model analysis with post-hoc Tukey test) as well as between week 2 and 5 ($p < 0.05$). There was no significant difference between the mean HSV of the primary tumor compared to the metastatic lymph nodes at baseline (9.5 vs. 4.1 mL, $p = 0.16$, paired t -test). The reduction of the tumoral HSV between week 0 and 5 was observed both in the primary GTV ($p < 0.05$) and in the nodal GTVs ($p < 0.05$) (Fig. 1A). A total of 5 patients had a maximum ^{18}F]FMISO SUV tumor-to-muscle ratio of ≤ 1.4 in week 2 of chemoradiation. At this time point, 11 patients exhibited an HSV of <1 mL, while 16 still had an HSV of ≥ 1 mL. Of the 11 patients with HSV <1 mL, 6 had an HSV = 0 mL. Patients with HPV-positive HNSCCs exhibited significantly more often an HSV <1 mL after 2 weeks of chemoradiation than patients with HPV-

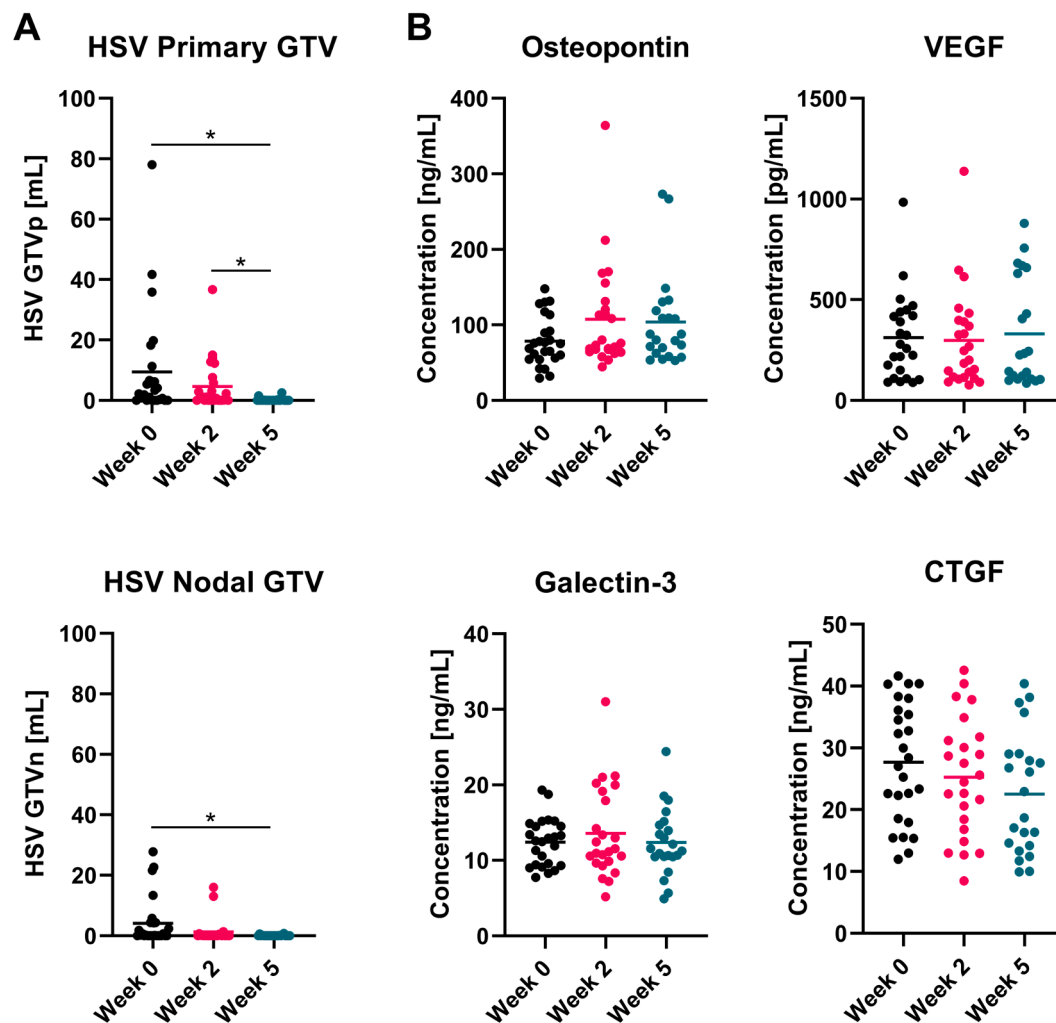


Fig. 1. Chemoradiation reduces the HSV in the primary tumor and in the metastatic lymph nodes. (A) HSV within the primary tumor (GTVp) and within the metastatic lymph nodes (GTVn) over the course of treatment. (B) Plasma concentration of osteopontin, galectin-3, VEGF and CTGF in week 0, 2 and 5 of chemoradiation. Values were compared using a mixed model analysis with post-hoc Tukey tests. * $p < 0.05$. CTGF = connective tissue growth factor, GTV = gross tumor volume, HSV = hypoxic subvolume, VEGF = vascular endothelial growth factor.

negative tumors ($p < 0.05$, chi-square test). In week 5 of treatment, nine patients still had residual hypoxia (HSV > 0 mL), either in the primary GTV ($n = 5$) or in the nodal GTVs ($n = 4$).

Mean plasma levels for osteopontin, galectin-3, VEGF and CTGF amounted to 78.6 ± 32.8 ng/mL, 12.4 ± 3.1 ng/mL, 306.7 ± 214.0 pg/mL and 27.7 ± 9.6 ng/mL at baseline, respectively (Fig. 1B). There was a trend towards increasing osteopontin plasma levels over the course of treatment ($p = 0.07$), whereas CTGF plasma levels exhibited a trend towards decreased plasma concentrations ($p = 0.08$). Neither galectin-3 ($p = 0.14$) nor VEGF ($p = 0.84$) concentrations were found to significantly alter during chemoradiation.

Osteopontin and galectin-3 plasma levels correlate with PET-derived baseline hypoxia

Pearson's correlation analyses revealed a moderate relationship between baseline osteopontin and the HSV prior to chemoradiation ($r = 0.579$, $p < 0.01$) (Fig. 2A). The HSV also significantly correlated with the total tumor volume at baseline ($r = 0.661$, $p < 0.001$). A multiple linear regression analysis incorporating several clinical and tumor-related parameters (HSV, smoking, tumor and nodal stage, HPV status and gender) demonstrated that HSV was the only significant parameter for osteopontin at baseline ($\beta = 0.643$, $p < 0.01$) (Table 1). Patients with no

relevant hypoxia (HSV < 1 mL) at baseline ($n = 6$) showed a trend towards decreased osteopontin levels compared to patients with hypoxic tumors (55.3 vs. 84.5 ng/mL, $p = 0.07$). Similar to osteopontin, galectin-3 moderately correlated with the HSV at baseline ($r = 0.429$, $p < 0.05$). However, none of the tested markers was significantly associated with the HSV in week 2 of chemoradiation; only VEGF in week 2 showed a non-significant moderate relationship with the HSV at this time point ($r = 0.391$, $p = 0.07$) (Fig. 2B). We also analyzed potential associations between the total tumor volume and the different hypoxia plasma markers: Both baseline osteopontin ($r = 0.593$, $p < 0.01$) and CTGF plasma concentrations ($r = 0.436$, $p < 0.05$) correlated with the initial tumor volume, while there was no such association for VEGF ($r = 0.249$, $p = 0.24$) and galectin-3 ($r = 0.286$, $p = 0.17$) (Table 2S). When tumor and nodal stages were replaced by tumor volume in the multiple linear regression analysis, no variable remained a significant parameter regarding baseline osteopontin plasma concentration (Table 3S).

Residual hypoxia in week 2 of chemoradiation is associated with increased plasma levels of VEGF and CTGF at the end of treatment

As early peritherapeutic hypoxia dynamics and especially residual hypoxia in week 2 of treatment have been shown to be the most important prognosticators for HNSCC patients undergoing definitive

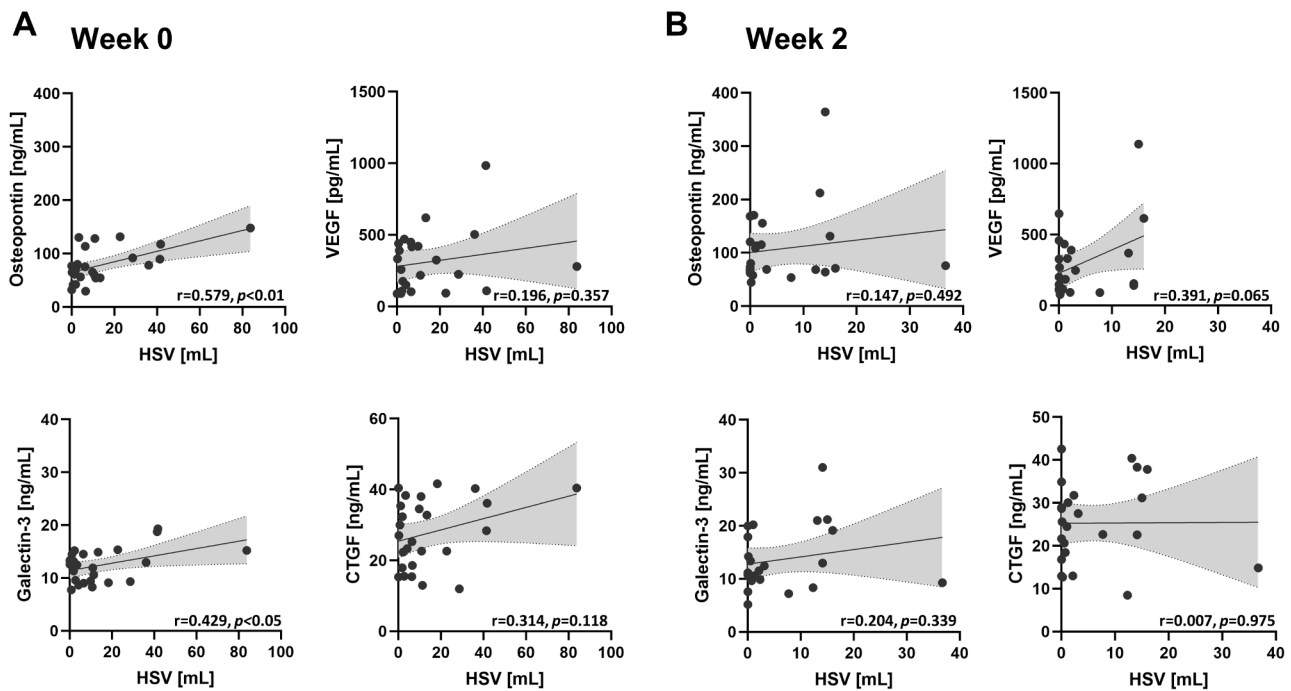


Fig. 2. Osteopontin and galectin-3 correlate with the tumoral HSV. Correlative analyses between HSV and different blood hypoxia markers in week 0 (A) and week 2 (B) of treatment. Pearson's r values with the according p -values are presented. In addition, regression lines with the corresponding 95% CI are shown in the diagrams.

Table 1

Multiple linear regression analysis with the dependent variable = osteopontin week 0. HPV = human papillomavirus, HSV = hypoxic subvolume, wk = week.

Variable	Standardized β	p
Gender	-0.187	0.346
Smoking during radiotherapy	-0.094	0.713
T stage	-0.258	0.266
N stage	0.167	0.405
HPV status	-0.199	0.410
HSV wk0 (continuous)	0.643	0.006

chemoradiation [21,37–39], we compared the hypoxia plasma markers in dependence of the HSV in week 2 (Fig. 3A). While there were no differences for osteopontin and galectin-3 depending on the HSV in week 2, both VEGF (451.1 vs. 221.7 ng/mL, $p < 0.05$) and CTGF (17.3 vs. 27.8 ng/mL, $p < 0.05$) in week 5 were about twice as high in patients with an HSV >1 mL at this time point. However, for a cut-off value of 0 mL, there were no significant differences in the plasma concentration of the analyzed markers.

Baseline osteopontin is increased in patients with residual PET-based hypoxia at the end of chemoradiation

As the tumor hypoxia status in week 5 has also been shown to be prognostic and may influence the overall tumor response to chemoradiation [38], we examined whether plasma hypoxia marker could predict residual hypoxia in week 5. Interestingly, baseline osteopontin was considerably higher in patients with residual hypoxia at week 5 of treatment (104.7 vs. 60.8 ng/mL, $p < 0.05$) (Fig. 3B). The AUC value of the ROC analysis regarding osteopontin-based residual hypoxia prediction was 0.821 (95% CI 0.604–1.000, $p < 0.05$), and the sensitivity and specificity for residual hypoxia prediction were 66.7% and 92.3%, respectively, for a cut-off value of 84.7 ng/mL (Youden index = 0.59) When using a cut-off value of 67.0 ng/mL for osteopontin plasma concentration, sensitivity for residual hypoxia prediction increased to

88.9%, while specificity decreased to 69.2% (Youden index = 0.58).

Plasma hypoxia markers do not correlate with LRC and PFS

LRC, PFS and OS amounted to 54.3%, 41.5% and 82.5% after 2 years. The prognostic values of osteopontin, galectin-3, VEGF and CTGF for LRC, PFS and OS were tested using Cox regression analyses (Table 2). While osteopontin, galectin-3 and VEGF did not exhibit prognostic value in our cohort, there was a trend towards impaired OS with increasing CTGF plasma concentrations both in week 0 (HR = 1.062, 95% CI 0.992–1.136, $p = 0.08$) and in week 2 (HR = 1.097, 95% CI 1.011–1.191, $p < 0.05$). However, CTGF concentrations in weeks 0 and 2 were no risk factor for LRC (week 0: HR = 1.061, 95% CI 0.983–1.146, $p = 0.13$, week 2: HR = 0.997, 95% CI 0.919–1.081, $p = 0.94$) and PFS (week 0: HR = 1.047, 95% CI 0.984–1.114, $p = 0.14$, week 2: HR = 1.050, 95% CI 0.983–1.122, $p = 0.15$).

Discussion

In this analysis of a prospective trial, we could demonstrate that both osteopontin and galectin-3 correlated with the baseline HSV and could therefore indicate more hypoxic tumors before radiotherapy initiation. Residual tumor hypoxia in week 2 of chemoradiation, known to be an important prognostic factor, resulted in increased VEGF and CTGF plasma levels at the end of treatment. Furthermore, baseline osteopontin was associated with residual tumor hypoxia at the end of treatment, another detrimental prognostic parameter for HNSCC patients undergoing chemoradiation.

Osteopontin is a bone sialoprotein that is involved in osteoclast attachment to mineralized bone matrix. Besides its role as non-collagenous bone matrix protein, osteopontin takes part in several pathways contributing to cancer progression such as proliferation, angiogenesis, epithelial-mesenchymal transition, metastasis and immunosuppression [40]. Osteopontin has been shown to correlate with tumor hypoxia in HNSCC both *in vitro* and *in vivo* [13,41–43]. Nordmark et al. could show in 67 HNSCC patients that plasma osteopontin

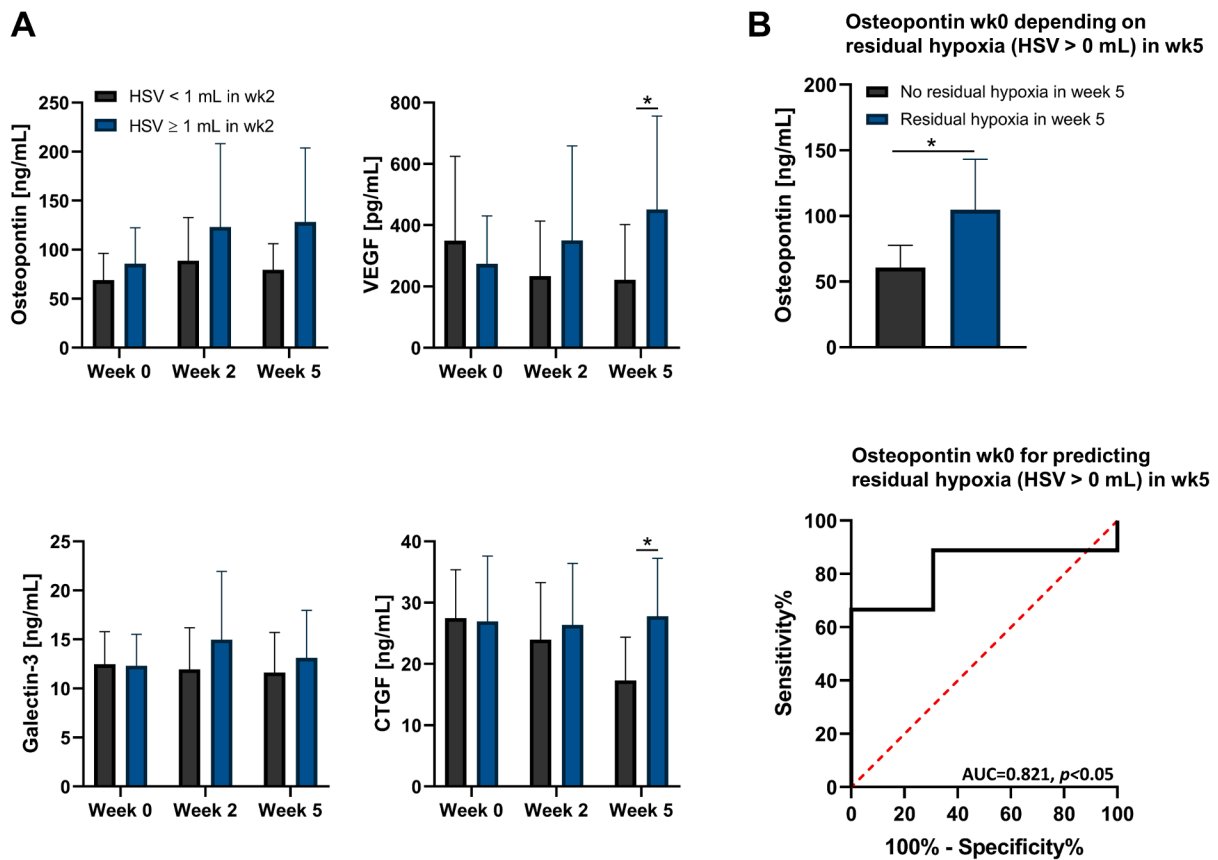


Fig. 3. Baseline osteopontin is associated with residual hypoxia in week 5 of chemoradiation. (A–B) Concentration of blood hypoxia markers in dependence of an HSV ≥ 1 mL in week 2 of chemoradiation. Data are given as mean \pm standard deviation, and groups were compared using unpaired t-tests. * $p < 0.05$. (B) ROC analyses of baseline osteopontin plasma values in terms of residual hypoxia (>0 mL) prediction in week 5. The AUC with the corresponding 95% CI and p -value is presented.

Table 2

Cox proportional hazards regression analyses for different blood hypoxia markers in terms of LRC, PFS and OS. CI = confidence interval, CTGF = connective tissue growth factor, HR = Hazard ratio, LRC = locoregional control, OS = overall survival, PFS = progression-free survival, VEGF = vascular endothelial growth factor, wk = week.

	LRC			PFS			OS		
	HR	95% CI	p	HR	95% CI	p	HR	95% CI	p
Osteopontin wk0	0.994	0.972–1.017	0.605	0.992	0.973–1.011	0.390	1.009	0.992–1.027	0.302
Osteopontin wk2	1.001	0.993–1.010	0.785	1.002	0.996–1.008	0.441	1.004	0.998–1.010	0.203
Osteopontin wk5	0.996	0.982–1.009	0.527	0.998	0.988–1.008	0.671	1.005	0.996–1.014	0.268
Galectin-3 wk0	0.793	0.615–1.023	0.074	0.856	0.701–1.046	0.128	0.922	0.726–1.170	0.503
Galectin-3 wk2	0.932	0.809–1.074	0.330	1.003	0.923–1.089	0.952	1.024	0.933–1.124	0.619
Galectin-3 wk5	0.913	0.749–1.112	0.365	0.977	0.854–1.118	0.734	1.003	0.856–1.174	0.973
VEGF wk0	1.001	0.997–1.004	0.786	1.002	0.999–1.006	0.186	1.001	0.996–1.005	0.739
VEGF wk2	0.998	0.994–1.002	0.370	0.999	0.996–1.002	0.497	0.999	0.995–1.003	0.506
VEGF wk5	1.000	0.997–1.002	0.869	1.000	0.998–1.002	0.992	1.000	0.997–1.003	0.964
CTGF wk0	1.061	0.983–1.146	0.130	1.047	0.984–1.114	0.143	1.062	0.992–1.136	0.082
CTGF wk2	0.997	0.919–1.081	0.937	1.050	0.983–1.122	0.146	1.097	1.011–1.191	0.027
CTGF wk5	1.012	0.938–1.092	0.757	1.005	0.944–1.069	0.887	1.048	0.977–1.125	0.191

inversely correlated with median tumor oxygen partial pressure (pO₂) [43]. In a post-hoc analysis of the DAHANCA 5 trial, Overgaard et al. demonstrated that higher plasma osteopontin levels were associated with a poor prognosis in HNSCC patients undergoing radiotherapy. However, it was also shown that this poor prognosis can be significantly improved when adding the hypoxic sensitizer nimorazole simultaneously to radiotherapy [13]. Osteopontin may therefore serve as predictor for patients that could benefit from hypoxic modification. In our study, baseline osteopontin was almost twice as high in patients with residual hypoxia in week 5 and may predict residual hypoxia at the end

of chemoradiation. In this context, Löck et al. have shown that tumor hypoxia in week 5 is an unfavorable prognosticator, although absent hypoxia response in week 2 was found to be a more important parameter in most studies [37,39]. The strong association between baseline hypoxia and residual tumor hypoxia at the end of chemoradiation may explain why pretreatment osteopontin was a predictor for poor response to radiotherapy in a previous study [30]. A potential clinical implication derived from these observations could be that patients with higher osteopontin concentrations prior to chemoradiation may benefit from hypoxia-based radiotherapy escalation approaches, e.g., hypoxic

modification or radiation dose escalation to the residual HSVs during treatment [44]. The observation regarding the correlation between osteopontin and the total tumor volume is in line with previous studies: Two previous publications demonstrated an association in oropharyngeal and nasopharyngeal cancers [45,46]. In the study of Snitcovsky and coworkers, higher osteopontin plasma levels were associated with advanced T and N stages in HNSCC patients undergoing chemoradiation [47]. In a mouse mammary carcinoma model, osteopontin plasma levels were found to rise with increasing tumor volumes [48]. These studies of course raise the question whether our observations concerning the association between osteopontin plasma levels and the HSV as well as hypoxia resolution are rather related to the tumor volume itself with the HSV only serving as confounder variable. However, there are several preclinical studies showing elevated osteopontin expression and secretion in hypoxic conditions, making our observations plausible [41,49,50]. Even though, due to the also significant correlation between the total tumor volume and the HSV in our cohort, it cannot completely be resolved to what extent both parameters contribute to osteopontin plasma levels.

Furthermore, the increase of osteopontin plasma levels during chemoradiation could be related to confounding variables such as inflammation [51], weight loss [52], mucositis [53], or irradiation itself [42]. As many of the above-mentioned parameters become more relevant during the course of chemoradiation, it is plausible that the hypoxic tumor volume significantly correlates with osteopontin levels at baseline but not during treatment. Many of the confounding variables are especially relevant in HNSCC, potentially explaining the discrepancy regarding osteopontin kinetics between HNSCC and other tumor types [54,55]. In this respect, previous publications demonstrated incongruent findings about the dynamics of plasma osteopontin levels during the course of treatment [32,47].

Members of the galectin family, a group of proteins that bind to β -galactose residues and regulate several biological functions such as proliferation, adhesion, migration and invasion, have also been linked with tumor hypoxia, providing a rationale to analyze the relationship between [18 F]FMISO PET-based hypoxia and galectin-3 levels [31,33]. Plasma galectin-3 in pathologic concentrations has been reported to induce secretion of metastasis-promoting cytokines such as interleukin-6, that also is up-regulated by hypoxia [56,57], from blood vascular endothelial cells both *in vitro* and *in vivo* [58]. As galectin-3 is induced by hypoxia and can capture interferon- γ in the tumor, resulting in impaired T-cell tumor infiltration, galectin-3 is a protein that links tumor hypoxia with tumor immune suppression [59,60]. To the best of our knowledge, we could show for the first time a moderate but significant correlation between galectin-3 plasma levels and the HSV at baseline in patients with locally advanced HNSCCs. The association between galectin-3 and tumor hypoxia could at least partly explain the prognostic role of both circulating and cytoplasmatic galectin-3 in HNSCC patients [61,62].

CTGF is a multifunctional signaling modulator that takes part in cancer progression and metastasis [63]. CTGF levels are increased by hypoxia both in normal and in tumor cells [64], and CTGF has been found to be prognostic in HNSCC patients [65]. In contrast to osteopontin and galectin-3, CTGF plasma levels did not correlate with tumor hypoxia at any of the tested time points in our study. This is in divergence to a previous study in which CTGF tissue expression (determined using immunohistochemistry stainings) correlated with the tissue hypoxia marker carbonic anhydrase IX, although correlative analyses between CTGF and pO₂ were not performed [33]. As CTGF plasma levels have been shown to be influenced by several diseases such as diabetes, chronic heart failure and chronic liver diseases [66–68], a potentially weak association between tumor hypoxia and CTGF plasma levels may be remained unnoticed and can only be revealed using prospective trials with larger patient numbers, such as the currently ongoing [18 F]FMISO-based de-escalation trials for patients with HPV-positive oropharyngeal carcinoma (NCT00606294, NCT03323463) [69].

VEGF is a proangiogenic cytokine that promotes proliferation and migration of endothelial cells and increases vessel permeability. As VEGF expression is induced by the hypoxia inducible factor 1 (HIF-1), VEGF could serve as indirect hypoxia marker [70]. In line with a previous study analyzing the interaction between [18 F]flortanidazole ([18 F]HX4)-PET imaging-based hypoxia and VEGF plasma levels, we could not detect a significant correlation between the HSVs and VEGF, although there was a trend for a moderate-to-weak correlation in week 2 [32]. In glioblastoma and soft tissue sarcoma, VEGF tissue expression was weakly associated with the [18 F]FMISO tumor SUV or HSV, respectively [71,72]. Although VEGF levels have been demonstrated to be higher in HNSCC patients compared to healthy controls and correlated with the pO₂ in one study, our analysis could not show a significant correlation between [18 F]FMISO-measured tumor hypoxia and VEGF plasma levels [73,74].

Despite the fact that our data are generated from a prospective trial, there are some limitations of our study. Although samples were stored at -80°C , plasma concentrations may have decreased over the time. Even though plasma hypoxia marker concentrations are dependent on the choice of the commercial ELISA system, the plasma concentrations measured in our study were in the range reported in previous studies [13,32,61,75,76]. Furthermore, our study has a limited sample size, and we did not correct for multiple testing, as this was an exploratory hypothesis-generating analysis. As discussed above, treatment-related parameters such as systemic inflammation, weight loss, mucositis and radiotherapy itself may have influenced some of the parameters, complicating correlative analyses between tumor hypoxia and the analyzed hypoxia plasma markers. Due to these limitations, our results should be interpreted cautiously. Validation is therefore mandatory to confirm our observations and to draw definitive conclusions.

Conclusion

Baseline osteopontin and galectin-3 plasma levels moderately correlate with the initial HSV, and increased osteopontin is associated with a higher likelihood of residual tumor hypoxia at the end of treatment. Our data show a promising role of osteopontin as a plasma hypoxia marker that may facilitate hypoxia-based personalized radiotherapy concepts, e.g., by concomitant treatment with the hypoxia modifier nimorazole. However, as plasma hypoxia markers do not provide information regarding the spatial distribution of tumor hypoxia they cannot replace [18 F]FMISO PET/CT-imaging for localized personalization approaches, e.g., for hypoxia-directed focal radiotherapy dose escalation. Further studies are needed to validate our findings and to fully explore the potential of plasma hypoxia markers for radiotherapy personalization approaches.

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.

Acknowledgements

The authors thank Dr. Andrei Bunea, Dr. Hatice Bunea and Dr. Eleni Christofi for their support regarding patient recruitment.

Alexander Rühle was supported by the IMM-PACT Programme for Clinician Scientists, Department of Medicine II, Medical Center – University of Freiburg and Faculty of Medicine, University of Freiburg, funded by the Deutsche Forschungsgemeinschaft (German Research Foundation, project no. 413517907). Parts of this trial were funded by a grant of the Deutsche Forschungsgemeinschaft to Nils Nicolay (German Research Foundation, project no. 443978314) and by the German Cancer Consortium (DKTK).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ctro.2022.02.008>.

References

- [1] Braakhuis BJM, Leemans CR, Visser O. Incidence and survival trends of head and neck squamous cell carcinoma in the Netherlands between 1989 and 2011. *Oral Oncol.* 2014;50(7):670–5.
- [2] Svahn MF, Munk C, Nielsen TSS, von Buchwald C, Frederiksen K, Kjaer SK. Trends in all-cause five-year mortality after head and neck cancers diagnosed over a period of 33 years. Focus on estimated degree of association with human papillomavirus. *Acta Oncol* 2016;55(9-10):1084–90.
- [3] Bernier J, Dommange C, Ozsahin M, Matuszewska K, Lefèbvre J-L, Greiner RH, et al. Postoperative Irradiation with or without Concomitant Chemotherapy for Locally Advanced Head and Neck Cancer. *N Engl J Med* 2004;350(19):1945–52.
- [4] Cooper JS, Pajak TF, Forastiere AA, Jacobs J, Campbell BH, Saxman SB, et al. Postoperative Concurrent Radiotherapy and Chemotherapy for High-Risk Squamous-Cell Carcinoma of the Head and Neck. *N Engl J Med* 2004;350(19):1937–44.
- [5] Adelstein DJ, Li Yi, Adams GL, Wagner H, Kish JA, Ensley JF, et al. An intergroup phase III comparison of standard radiation therapy and two schedules of concurrent chemoradiotherapy in patients with unresectable squamous cell head and neck cancer. *J Clin Oncol.* 2003;21(1):92–8.
- [6] Nichols AC, Theurer J, Prisman E, Read N, Berthelet E, Tran E, et al. Radiotherapy versus transoral robotic surgery and neck dissection for oropharyngeal squamous cell carcinoma (ORATOR): an open-label, phase 2, randomised trial. *Lancet Oncol* 2019;20(10):1349–59.
- [7] Lacas B, Carmel A, Landais C, Wong SJ, Licitra L, Tobias JS, et al. Meta-analysis of chemotherapy in head and neck cancer (MACH-NC): An update on 107 randomized trials and 19,805 patients, on behalf of MACH-NC Group. *Radiother Oncol.* 2021;156:281–93.
- [8] Mortensen LS, Johansen J, Kallehauge J, Primdahl H, Busk M, Lassen P, et al. FAZA PET/CT hypoxia imaging in patients with squamous cell carcinoma of the head and neck treated with radiotherapy: Results from the DAHANCA 24 trial. *Radiother Oncol* 2012;105(1):14–20.
- [9] Vauppel P, Thews O, Hoecckel M. Treatment resistance of solid tumors. *Med Oncol* 2001;18(4):243–60.
- [10] Gray LH, Conger AD, Ebert M, Hornsey S, Scott OCA. The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy. *Br J Radiol.* 1953;26(312):638–48.
- [11] Linge A, Löck S, Gudziol V, Nowak A, Lohaus F, von Neubeck C, et al. Low Cancer Stem Cell Marker Expression and Low Hypoxia Identify Good Prognosis Subgroups in HPV(-) HNSCC after Postoperative Radiochemotherapy: A Multicenter Study of the DTKK-ROG. *Clin Cancer Res.* 2016;22(11):2639–49.
- [12] Zips D, Böke S, Kroeber T, Meinzer A, Brüchner K, Thames HD, et al. Prognostic value of radiobiological hypoxia during fractionated irradiation for local tumor control/Prognostischer Wert der radiobiologischen Hypoxie während fraktionierter Bestrahlung für die lokale Tumorkontrolle. *Strahlenther Onkol.* 2011;187(5):306–10.
- [13] Overgaard J, Eriksen JG, Nordmark M, Alsner J, Horsman MR. Plasma osteopontin, hypoxia, and response to the hypoxia sensitizer nimorazole in radiotherapy of head and neck cancer: results from the DAHANCA 5 randomised double-blind placebo-controlled trial. *Lancet Oncol* 2005;6(10):757–64.
- [14] Janssens GO, Rademakers SE, Terhaard CH, Doornaert PA, Bijl HP, van den Ende P, et al. Accelerated radiotherapy with carbogen and nicotinamide for laryngeal cancer: results of a phase III randomized trial. *J Clin Oncol.* 2012;30(15):1777–83.
- [15] Overgaard J. Hypoxic modification of radiotherapy in squamous cell carcinoma of the head and neck—a systematic review and meta-analysis. *Radiother Oncol.* 2011;100(1):22–32.
- [16] Welz S, Mönlich D, Pfannenber C, Nikolaou K, Reimold M, La Fougère C, et al. Prognostic value of dynamic hypoxia PET in head and neck cancer: Results from a planned interim analysis of a randomized phase II hypoxia-image guided dose escalation trial. *Radiother Oncol.* 2017;124(3):526–32.
- [17] Grosu A-L, Souvatzoglou M, Röper B, Dobritz M, Wiedenmann N, Jacob V, et al. Hypoxia imaging with FAZA-PET and theoretical considerations with regard to dose painting for individualization of radiotherapy in patients with head and neck cancer. *Int J Radiat Oncol Biol Phys.* 2007;69(2):541–51.
- [18] Zips D, Le K, Yaromina A, Dörfler A, Eicheler W, Zhou X, et al. Triple angiokinase inhibition, tumour hypoxia and radiation response of FaDu human squamous cell carcinomas. *Radiother Oncol.* 2009;92(3):405–10.
- [19] Thorwarth D, Eschmann S-M, Paulsen F, Alber M. Hypoxia dose painting by numbers: a planning study. *Int J Radiat Oncol Biol Phys.* 2007;68(1):291–300.
- [20] Riaz N, Sherman E, Pei X, Schöder H, Grkovski M, Paudyal R, et al. Precision Radiotherapy: Reduction in Radiation for Oropharyngeal Cancer in the 30 ROC Trial. *J Natl Cancer Inst.* 2021;113(6):742–51.
- [21] Lee N, Schoder H, Beattie B, Lanning R, Riaz N, McBride S, et al. Strategy of Using Intra-treatment Hypoxia Imaging to Selectively and Safely Guide Radiation Dose De-escalation Concurrent With Chemotherapy for Locoregionally Advanced Human Papillomavirus-Related Oropharyngeal Carcinoma. *Int J Radiat Oncol Biol Phys.* 2016;96(1):9–17.
- [22] Rajendran JG, Schwartz DL, O’Sullivan J, Peterson LM, Ng P, Scharnhorst J, et al. Tumor hypoxia imaging with [F-18] fluoromisonidazole positron emission tomography in head and neck cancer. *Clin Cancer Res.* 2006;12(18):5435–41.
- [23] Rasey JS, Koh W-J, Evans ML, Peterson LM, Lewellen TK, Graham MM, et al. Quantifying regional hypoxia in human tumors with positron emission tomography of [18F]fluoromisonidazole: a pretherapy study of 37 patients. *Int J Radiat Oncol Biol Phys.* 1996;36(2):417–28.
- [24] Zschaek S, Löck S, Hofheinz F, Zips D, Sakso Mortensen L, Zöphel K, et al. Individual patient data meta-analysis of FMISO and FAZA hypoxia PET scans from head and neck cancer patients undergoing definitive radio-chemotherapy. *Radiother Oncol.* 2020;149:189–96.
- [25] Sanduleanu S, Hamming-Vrieze O, Wesseling FWR, Even AJG, Hoebers FJ, Hoeben A, et al. [(18)F]-HX4 PET/CT hypoxia in patients with squamous cell carcinoma of the head and neck treated with chemoradiotherapy: Prognostic results from two prospective trials. *Clin Transl Radiat Oncol.* 2020;23:9–15.
- [26] Toustrup K, Sorensen BS, Lassen P, Wiuf C, Alsner J, Overgaard J. Gene expression classifier predicts for hypoxic modification of radiotherapy with nimorazole in squamous cell carcinomas of the head and neck. *Radiother Oncol.* 2012;102(1):122–9.
- [27] Toustrup K, Sorensen BS, Metwally MAH, Tramm T, Mortensen LS, Overgaard J, et al. Validation of a 15-gene hypoxia classifier in head and neck cancer for prospective use in clinical trials. *Acta Oncol* 2016;55(9-10):1091–8.
- [28] Wiedenmann N, Grosu A-L, Büchert M, Rischke HC, Ruf J, Bielak L, et al. The utility of multiparametric MRI to characterize hypoxic tumor subvolumes in comparison to FMISO PET/CT. Consequences for diagnosis and chemoradiation treatment planning in head and neck cancer. *Radiother Oncol.* 2020;150:128–35.
- [29] Brøndum L, Eriksen JG, Singers Sørensen B, Mortensen LS, Toustrup K, Overgaard J, et al. Plasma proteins as prognostic biomarkers in radiotherapy treated head and neck cancer patients. *Clin Transl Radiat Oncol.* 2017;2:46–52.
- [30] Hui EP, Sung FL, Yu BKH, Wong CSC, Ma BBY, Lin X, et al. Plasma Osteopontin, Hypoxia, and Response to Radiotherapy in Nasopharyngeal Cancer. *Clin Cancer Res* 2008;14(21):7080–7.
- [31] Le Q-T, Shi G, Cao H, Nelson DW, Wang Y, Chen EY, et al. Galectin-1: a link between tumor hypoxia and tumor immune privilege. *J Clin Oncol.* 2005;23(35):8932–41.
- [32] Zegers CML, Hoebers FJP, van Elmpot W, Bons JA, Öllers MC, Troost EGC, et al. Evaluation of tumour hypoxia during radiotherapy using [(18)F]HX4 PET imaging and blood biomarkers in patients with head and neck cancer. *Eur J Nucl Med Mol Imaging* 2016;43(12):2139–46.
- [33] Le Q-T, Kong C, Lavori PW, O’Byrne K, Erler JT, Huang X, et al. Expression and prognostic significance of a panel of tissue hypoxia markers in head-and-neck squamous cell carcinomas. *Int J Radiat Oncol Biol Phys.* 2007;69(1):167–75.
- [34] Bennewit KL, Huang X, Ham CM, Graves EE, Erler JT, Kambham N, et al. The Role of Tumor Cell-Derived Connective Tissue Growth Factor (CTGF/CCN2) in Pancreatic Tumor Growth. *Cancer Res* 2009;69(3):775–84.
- [35] Carles M, Fechter T, Grosu AL, Sorensen A, Thomann B, Stoian RG, et al. 18F-FMISO-PET Hypoxia Monitoring for Head-and-Neck Cancer Patients: Radiomics Analyses Predict the Outcome of Chemo-Radiotherapy. *Cancers.* 2021;13(14):3449. <https://doi.org/10.3390/cancers13143449>.
- [36] Kroenke M, Hirata K, Gafita A, Watanabe S, Okamoto S, Magota K, et al. Voxel based comparison and texture analysis of 18F-FDG and 18F-FMISO PET of patients with head-and-neck cancer. *PLoS ONE* 2019;14(2):e0213111. <https://doi.org/10.1371/journal.pone.0213111>.
- [37] Nicolay NH, Rühle A, Wiedenmann N, Niedermann G, Mix M, Weber WA, et al. Lymphocyte Infiltration Determines the Hypoxia-Dependent Response to Definitive Chemoradiation in Head-and-Neck Cancer – Results from a Prospective Imaging Trial. *J Nucl Med.* 2021;62(4):471–8.
- [38] Löck S, Perrin R, Seidlitz A, Bandurska-Luque A, Zschaek S, Zöphel K, et al. Residual tumour hypoxia in head-and-neck cancer patients undergoing primary radiochemotherapy, final results of a prospective trial on repeat FMISO-PET imaging. *Radiother Oncol.* 2017;124(3):533–40.
- [39] Zips D, Zöphel K, Abolmaali N, Perrin R, Abramjuk A, Haase R, et al. Exploratory prospective trial of hypoxia-specific PET imaging during radiochemotherapy in patients with locally advanced head-and-neck cancer. *Radiother Oncol.* 2012;105(1):21–8.
- [40] Zhao H, Chen Q, Alam A, Cui J, Suen KC, Soo AP, et al. The role of osteopontin in the progression of solid organ tumour. *Cell Death Dis* 2018;9(3). <https://doi.org/10.1038/s41419-018-0391-6>.
- [41] Le QT, Sutphin PD, Raychaudhuri S, Yu SC, Terris DJ, Lin HS, et al. Identification of osteopontin as a prognostic plasma marker for head and neck squamous cell carcinomas. *Clin Cancer Res.* 2003;9:59–67.
- [42] Wohlleben G, Scherzad A, Güttler A, Vordermark D, Kuger S, Flentje M, et al. Influence of hypoxia and irradiation on osteopontin expression in head and neck cancer and glioblastoma cell lines. *Radiat Oncol.* 2015;10(1). <https://doi.org/10.1186/s13014-015-0473-x>.
- [43] Nordmark M, Eriksen JG, Gebski V, Alsner J, Horsman MR, Overgaard J. Differential risk assessments from five hypoxia specific assays: The basis for biologically adapted individualized radiotherapy in advanced head and neck cancer patients. *Radiother Oncol.* 2007;83(3):389–97.
- [44] Pigorsch SU, Wilkens JJ, Kampfer S, Kehl V, Hapfelmeier A, Schläger C, et al. Do selective radiation dose escalation and tumour hypoxia status impact the loco-regional tumour control after radio-chemotherapy of head & neck tumours? The ESCALOX protocol. *Radiation Oncology.* 2017;12:45.
- [45] Mardani M, Andisheh-Tadibir A, Khademi B, Fattahi MJ, Shafiee S, Asad-Zadeh M. Serum levels of osteopontin as a prognostic factor in patients with oral squamous cell carcinoma. *Tumour Biol.* 2014;35(4):3827–9.

- [46] Wong TS, Kwong D-L-W, Sham J, Wei W, Kwong YL, Yuen A-P-W. Elevation of plasma osteopontin level in patients with undifferentiated nasopharyngeal carcinoma. *Eur J Surg Oncol*. 2005;31(5):555–8.
- [47] Snitcovsky I, Leitão GM, Pasini FS, Brunialti KCS, Mangone FRR, Maistro S, et al. Plasma osteopontin levels in patients with head and neck cancer undergoing chemoradiotherapy. *Arch Otolaryngol Head Neck Surg*. 2009;135(8):807. <https://doi.org/10.1001/archoto.2009.103>.
- [48] Lukacova S, Khalil AA, Overgaard J, Alsner J, Horsman MR. Relationship between radiobiological hypoxia in a C3H mouse mammary carcinoma and osteopontin levels in mouse serum. *Int J Radiat Biol*. 2005;81(12):937–44.
- [49] Sodhi CP, Phadke SA, Batlle D, Sahai A. Hypoxia stimulates osteopontin expression and proliferation of cultured vascular smooth muscle cells: potentiation by high glucose. *Diabetes* 2001;50:1482–90.
- [50] Sørensen BS, Hao J, Overgaard J, Vorum H, Honoré B, Alsner J, et al. Influence of oxygen concentration and pH on expression of hypoxia induced genes. *Radiother Oncol*. 2005;76(2):187–93.
- [51] Castello LM, Baldrighi M, Molinari L, Salmi L, Cantaluppi V, Vaschetto R, et al. The Role of Osteopontin as a Diagnostic and Prognostic Biomarker in Sepsis and Septic Shock. *Cells*. 2019;8(2):174. <https://doi.org/10.3390/cells8020174>.
- [52] Karadag F, Gulen ST, Karul AB, Kilicarslan N, Ceylan E, Kuman NK, et al. Osteopontin as a marker of weight loss in lung cancer. *Scand J Clin Lab Invest*. 2011;71(8):690–4.
- [53] Sodek J, Batista Da Silva AP, Zohar R. Osteopontin and mucosal protection. *J Dent Res*. 2006;85(5):404–15.
- [54] Ostheimer C, Schweyer F, Reese T, Bache M, Vordermark D. The relationship between tumor volume changes and serial plasma osteopontin detection during radical radiotherapy of non-small-cell lung cancer. *Oncol Lett*. 2016;12:3449–56.
- [55] Xu C, Yuan Qi, Wang W, Chi C, Zhang Q, Li Li, et al. Prognostic significance of serum osteopontin levels in small cell lung cancer. *BMC Pulm Med*. 2020;20(1). <https://doi.org/10.1186/s12890-020-01242-3>.
- [56] A. Rühle N, Wiedenmann J.T, Fennell M, Mix J, Ruf R, Stoian et al. Interleukin-6 as surrogate marker for imaging-based hypoxia dynamics in patients with head-and-neck cancers undergoing definitive chemoradiation-results from a prospective pilot trial 10.1007/s00259-021-05602-x.
- [57] Klausen T, Olsen NV, Poulsen TD, Richalet J-P, Pedersen BK. Hypoxemia increases serum interleukin-6 in humans. *Eur J Appl Physiol* 1997;76(5):480–2.
- [58] Chen C, Duckworth CA, Zhao Q, Pritchard DM, Rhodes JM, Yu L-G. Increased Circulation of Galectin-3 in Cancer Induces Secretion of Metastasis-Promoting Cytokines from Blood Vascular Endothelium. *Clin Cancer Res* 2013;19(7):1693–704.
- [59] Zheng J, Lu W, Wang C, Xing Y, Chen X, Ai Z. Galectin-3 induced by hypoxia promotes cell migration in thyroid cancer cells. *Oncotarget*. 2017;8(60):101475–88.
- [60] Gordon-Alonso M, Hirsch T, Wildmann C, van der Bruggen P. Galectin-3 captures interferon-gamma in the tumor matrix reducing chemokine gradient production and T-cell tumor infiltration. *Nat Commun* 2017;8:793.
- [61] Saussez S, Lorfèvre F, Lequeux T, Laurent G, Chantrain G, Vertongen F, et al. The determination of the levels of circulating galectin-1 and -3 in HNSCC patients could be used to monitor tumor progression and/or responses to therapy. *Oral Oncol* 2008;44(1):86–93.
- [62] Honjo Y, Inohara H, Akahani S, Yoshii T, Takenaka Y, Yoshida J, et al. Expression of cytoplasmic galectin-3 as a prognostic marker in tongue carcinoma. *Clin Cancer Res*. 2000;6:4635–40.
- [63] Shen Y-W, Zhou Y-D, Chen H-Z, Luan X, Zhang W-D. Targeting CTGF in Cancer: An Emerging Therapeutic Opportunity. *Trends Cancer*. 2021;7(6):511–24.
- [64] Kondo S, Kubota S, Shimo T, Nishida T, Yosimichi G, Eguchi T, et al. Connective tissue growth factor increased by hypoxia may initiate angiogenesis in collaboration with matrix metalloproteinases. *Carcinogenesis* 2002;23:769–76.
- [65] Kikuchi R, Kikuchi Y, Tsuda H, Maekawa H, Kozaki K, Imoto I, et al. Expression and clinical significance of connective tissue growth factor in advanced head and neck squamous cell cancer. *Hum Cell*. 2014;27:121–8.
- [66] Roestenberg P, van Nieuwenhoven FA, Wieten L, Boer P, Diekman T, Tiller AM, et al. Connective Tissue Growth Factor Is Increased in Plasma of Type 1 Diabetic Patients With Nephropathy. *Diabetes Care* 2004;27(5):1164–70.
- [67] Koitabashi N, Arai M, Niwano K, Watanabe A, Endoh M, Suguta M, et al. Plasma connective tissue growth factor is a novel potential biomarker of cardiac dysfunction in patients with chronic heart failure. *Eur J Heart Fail* 2008;10(4):373–9.
- [68] Gressner OA, Fang M, Li H, Lu LG, Gressner AM, Gao CF. Connective tissue growth factor (CTGF/CCN2) in serum is an indicator of fibrogenic progression and malignant transformation in patients with chronic hepatitis B infection. *Clin Chim Acta*. 2013;421:126–31.
- [69] Rühle A, Grosu A-L, Nicolay NH. De-Escalation Strategies of (Chemo)Radiation for Head-and-Neck Squamous Cell Cancers—HPV and Beyond. *Cancers*. 2021;13(9):2204. <https://doi.org/10.3390/cancers13092204>.
- [70] Semenza G. Hypoxia-inducible factors in physiology and medicine. *Cell* 2012;148(3):399–408.
- [71] Rajendran JG, Wilson DC, Conrad EU, Peterson LM, Bruckner JD, Rasey JS, et al. [18F]FMISO and [18F]FDG PET imaging in soft tissue sarcomas: correlation of hypoxia, metabolism and VEGF expression. *Eur J Nucl Med Mol Imaging* 2003;30(5):695–704.
- [72] Kawai N, Lin W, Cao WD, Ogawa D, Miyake K, Haba R, et al. Correlation between ¹⁸F-fluoromisonidazole PET and expression of HIF-1 α and VEGF in newly diagnosed and recurrent malignant gliomas. *Eur J Nucl Med Mol Imaging*. 2014;41:1870–8.
- [73] Teknos TN, Cox C, Yoo S, Chepeha DB, Wolf GT, Bradford CR, et al. Elevated serum vascular endothelial growth factor and decreased survival in advanced laryngeal carcinoma. *Head Neck*. 2002;24(11):1004–11.
- [74] Dunst J, Stadler P, Becker A, Kuhnt T, Lautenschläger C, Molls M, et al. Tumor hypoxia and systemic levels of vascular endothelial growth factor (VEGF) in head and neck cancers. *Strahlenther Onkol*. 2001;177(9):469–73.
- [75] Riedel F, Gotte K, Schwalb J, Wirtz H, Bergler W, Hormann K. Serum levels of vascular endothelial growth factor in patients with head and neck cancer. *Eur Arch Otorhinolaryngol*. 2000;257(6):332–6.
- [76] Vordermark D, Said HM, Katzer A, Kuhnt T, Hänsgen G, Dunst J, et al. Plasma osteopontin levels in patients with head and neck cancer and cervix cancer are critically dependent on the choice of ELISA system. *BMC Cancer*. 2006;6(1). <https://doi.org/10.1186/1471-2407-6-207>.