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ORIGINAL ARTICLE

# Analysis of relation between hypoxia PET imaging and tissue-based biomarkers during head and neck radiochemotherapy

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**ABSTRACT**

**Background:** Tumor hypoxia is associated with poor prognosis and outcome and can be visualized using 18F-MISO-positron emission tomography (PET) imaging. The goal of this study was to evaluate the correlation between biological markers and biological imaging in a group of patients in whom a correlation between biological imaging and outcome has previously been demonstrated.

**Material and methods:** In a prospective pilot project, 16 patients with locally advanced cancer of the head and neck underwent 18F-MISO-PET scans before and during primary radiochemotherapy in addition to 18F-FDG-PET and computed tomography (CT). Tumor biopsies were stained for three tissue-based markers (Ku80, CAIX, CD44); in addition, human papillomavirus (HPV) status was assessed. H-scores of marker expression were generated and the results were correlated with the biological imaging and clinical outcome.

**Results:** No statistically significant correlation was established between the H-scores for Ku80, CD44 and CAIX or between any of the H-scores and the imaging variables (tumor volume on 18F-FDG-PET in ml, hypoxic subvolume as assessed by 18F-MISO-PET in ml, and SUVmax tumor/SUVmean muscle during the 18F-MISO-PET). A statistically significant negative correlation was found between CD44 H-score and HPV status ($p = .004$). Cox regression analysis for overall survival and recurrence-free survival showed one significant result for CAIX being associated with improved overall survival [hazard ratio 0.96 (0.93–1.00), $p = .047$].

**Conclusion:** Expression of Ku80, CAIX and CD44 as assessed by immunohistochemistry of tumor biopsies were not correlated to one another or the biological imaging data. However, there was a significant influence of CAIX on overall survival and between CD44 and HPV.

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Cancer of the head and neck is a frequent malignancy with a median five-year survival of around 50% [1]. One important prognostic factor is tumor hypoxia, which has been shown to be associated with reduced therapeutic effect of radiotherapy and decreased overall survival [2,3]. This is explained by decreased sensitivity towards radiation and reduced accessibility for chemotherapy [1,4].

Given the prognostic importance and the potential therapeutic consequences (e.g. alteration of radiotherapy, additional drugs), the analysis of tumor hypoxia has to be seen as an important research field. Novel imaging techniques, including biological imaging, can complement the information available for treatment planning so far and thus help to improve the therapeutic setting [5].

For hypoxia imaging, 18F-MISO-positron emission tomography (PET) is probably the most commonly used and best validated tracer so far [4,6]. The possibility and feasibility of using hypoxia imaging in a clinical setting in head and neck cancer patients – e.g. as a template for dose painting – has already been shown [7,8,9]. Good correlation of hypoxia PET with data generated using $pO_2$-polarography measurements in head and neck tumors have also been found [10]. In addition, there are recent works assessing the time course of hypoxia over treatment [3,11,12]. Previously, we and others showed a strong predictive capacity of hypoxia imaging in a group of patients with squamous cell carcinoma of the head and neck (HNSCC) receiving primary radiochemotherapy and undergoing serial 18F-MISO-PET scans for tumor hypoxia imaging in addition to 18F-FDG-PET [3,13].

However, the relation between tissue-based biomarkers and the information gathered from biological imaging are not well understood and there are only few studies addressing this issue (e.g. [14,15]).

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The goal of this study was to evaluate the correlation between select tissue-based biological markers and data obtained from PET imaging as well as outcome parameters in the same group of patients, and thus to elucidate the relation between:

1. The expression of the selected tissue-based biomarkers and human papillomavirus (HPV) status themselves;
2. The expression of tissue-based biomarkers and hypoxia & metabolic imaging;
3. The expression of tissue-based biomarkers and clinical outcome.

As tissue-based biomarkers, we chose carbonic anhydrase IX (CAIX) as cellular correlate for tumor hypoxia, CD44 as putative stem cell marker, Ku80 which is involved in DNA double-strandbreak repair and HPV, another well known prognostic factor in head and neck cancer patients.

The information gathered from these investigations was hypothesized to guide the use of the afore-mentioned tissue-based biomarkers in relation to the use of hypoxia imaging.

## Material and methods

### Cohort

Sixteen patients with HNSCC were included in this study. A detailed characterization of the study population is given elsewhere [13]. However, this study reports a cohort with an updated and prolonged follow-up. The primary tumor localizations (patient numbers in brackets) were oral cavity (one), oropharynx (seven), hypopharynx (five) and larynx (three). The patients were treated with primary radiochemotherapy; the protocol consisted of a total dose of 70 Gy in five fractions per week over seven weeks, and up to three concomitant cycles of cisplatin (Weeks 1, 4 and 7). The protocols for the imaging study and the subsequent tumor sample analysis have been approved by the Ethics Committee of the Medical Center – University of Freiburg (EK 68/08 and EK 296/12, respectively) and were in accordance with the ethical standards of the Helsinki Declaration of 1975, as revised in 1983. Written informed consent had been given by all patients for participation in the imaging study and has been waived for the subsequent tumor sample and correlation analysis.

### Imaging and radiotherapy

All patients received an 18F-FDG-PET scan, a planning computed tomography (CT) and magnetic resonance imaging (MRI) before starting the treatment. Up to three 18F-MISO-PET scans were performed: before starting the treatment and in weeks 2 and 5. For this analysis, the main focus was on the pretreatment scan because of the closest temporal proximity to the tumor biopsy. However, we also included the delta between the first and second as well as between the first and third 18F-MISO imaging timepoint to capture the development during therapy (see below for details). Scans were done with Siemens ECAT EXACT 921 PET scanner (15 patients) and Philips Gemini TrueFlight PET/CT scanner (one patient). The dose of 18F-MISO was 3.7 MBq/kg up to a maximum of 370 MBq; image acquisition started 150 minutes after tracer injection. Scan duration was 35 minutes (3 frames at 10 minutes, followed by a 5 minutes transmission scan). Having acquired the 18F-MISO-PET scans, these were co-registered with the CT scans (for the stand-alone ECAT scanner). Co-registration was done at the Siemens Syngo workstation using a mutual information algorithm (only translation and rotation), saving all co-registered PET scans in the coordinates of the planning CT. All imaging met standard clinical quality criteria, was validated, and performed with patients immobilized as for radiotherapy treatment using a head and neck mask. For quantitative analysis, three attenuation corrected frames were summed after excluding frames with patient movement [13].

Gross tumor volume (GTV) was defined according to the 18F-FDG-PET scan; an uptake exceeding 40% of the maximum standardized uptake value (SUV) was set as threshold and the resulting volume was validated with the CT and MRI data by an experienced clinician judging clinically acceptable quality of co-registration and image alignment as well as accuracy of the volume delineation. Hypoxic subvolumes were defined as all voxels within the GTV with a ratio of SUV to mean SUV in the contralateral neck musculature of $\geq$1.4. A ratio of 1.4 was chosen in accordance with the literature and after successfully validating its applicability in this patient cohort before [6,11]. The imaging variables used for the correlation analysis were the tumor volume on 18F-FDG-PET measured in milliliters, the hypoxic subvolumes as assessed by the 18F-MISO-PET measured in milliliters, and the ratio of SUVmax of the tumor and SUVmean in muscle during the first, second and third 18F-MISO-PET scan (equals the maximum SUV value in the tumor after normalization to muscle). The delta values between the first and second and the first and third scan were calculated by subtracting the respective normalized SUVmax tu values from one another.

### Immunohistochemistry

Tumor biopsies were taken by head and neck surgeons 4–6 weeks before initiation of treatment to confirm diagnosis. Tumor biopsies were analyzed for marker expression and correlation analyses were performed. All tumor samples were formalin fixed and paraffin-embedded using routine protocols. 2 μm sections of tumor biopsies were mounted on coated glass slide (Superfrost Plus, Langenbrinck), deparaffinized and rehydrated using a descending alcohol row. Heat-induced antigen retrieval was performed using citrate buffer at pH 6.1 for 40 minutes. After inhibition of endogenous peroxidase by $H_2O_2$ (5 minutes) primary antibodies (CAIX: Cell Signaling, monoclonal rabbit anti-human 1:100; CD44: Cell Signaling, monoclonal mouse anti-human 1:500; Ku80: Cell Signaling, rabbit anti-human, 1:300) were incubated for 30 minutes. Primary antibody detection and visualization was performed with the DAKO FLEX EnVision Kit using the rabbit linker (CAIX and Ku80) and mouse linker (CD44) for 15 minutes followed by horseradish peroxidase (20 minutes)

Table 1. Patient characteristics, imaging data (pretreatment 18F-FDG-PET (FDG) and 18F-MISO-PET (MISO)) and cumulative H-score for immunohistochemistry staining for CAIX, Ku80 and CD44 as well as results of HPV chip analysis for Patients 1–16.

| Age and gender | TNM | Follow-up (months) | Local failure | Death | Volume FDG (ml) | Volume MISO (ml) | Baseline MISO normalized SUVmax tu | CD44 - H-score | Ku80 - H-score | CAIX - H-score | HPV chip positive |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 64, M | T4 N2b | 77.1 | | | 26 | 7.4 | 2.2 | 300 | 180 | 15 | 0 |
| 52, M | T4 N2c | 7.3 | recurrence | tumor | 20 | 14.9 | 2.4 | 300 | 210 | 5 | 0 |
| 61, F | T4 N2c | 0 | no remission | tumor | 99 | 73.8 | 2.4 | 280 | 260 | 25 | 0 |
| 75, F | T2 N2c | 0.6 | | other | 24 | 4.7 | 1.6 | 280 | 240 | 20 | 0 |
| 62, M | T4 N2c | 35.1 | | second malignancy | 10 | 1.9 | 1.5 | 285 | 230 | 30 | 0 |
| 48, M | T4 N2c | 6.3 | recurrence | tumor | 29 | 17.2 | 2.4 | 300 | 270 | 45 | 0 |
| 68, F | T3 N2c | 73.7 | | | 11 | 10.3 | 1.7 | 250 | 270 | 0 | 1 |
| 47, M | T4 N2c | 12.3 | | metastasis | 20 | 15 | 1.6 | 280 | 170 | 10 | 0 |
| 71, M | T4 N2c | 62.3 | | | 16 | 12.2 | 1.7 | 270 | 200 | 90 | 0 |
| 69, M | T3 N2c | 75.3 | | | 9 | 5.3 | 2.3 | 280 | 210 | 5 | 0 |
| 66, M | T3 N2c | 44 | recurrence | | 16 | 15.8 | 2.3 | 280 | 200 | 130 | 0 |
| 51, M | T3 N2c | 5.9 | | other | 25 | 2.9 | 1.5 | 295 | 240 | 5 | 0 |
| 44, M | T4 N2b | 73.1 | | | 22 | 1 | 1.1 | 300 | 220 | 75 | 0 |
| 61, M | T4 N2c | 66.5 | | | 35 | 19.8 | 1.9 | 270 | 120 | 65 | 1 |
| 51, M | T4 N2c | 63.1 | | | 62 | 42 | 2.2 | 300 | 280 | 120 | 0 |
| 44, M | T4 N2c | 8.3 | recurrence | tumor | 15 | 8 | 2.1 | 285 | 280 | 15 | 0 |

and diaminobenzidine (10 minutes). All slides were counterstained by hematoxylin, dehydrated in an ascending alcohol row and coverslipped.

For microscopic analysis, all slides were digitized using a Panoramic Scan (3D Histech, Hungary). Visual assessment was performed following the H-score method. Briefly, the intensity of staining is scored in three grades and multiplied by the respective percentage of positive tumor cells: 3× percentage of strongly stained cells +2× percentage of moderately stained cells +1× percentage of weakly stained cells +0× percentage of negative cells, yielding a final score of 0–300. This technique has been described in detail before [16]. Only specific staining patterns for each marker were taken into account, i.e. membranous for CD44 and CAIX and nuclear for Ku80.

### *HPV-analysis*

After microdissection of tumor tissue from marked histological slides, DNA was isolated using routine protocols. For detection of HPV-specific DNA the Chipron HPV 3.5 LCD array kit (Chipron GmbH, Berlin, Germany) was used according to the supplier's instructions, yielding a binary score. Positive and negative controls were performed for each run.

### *Statistical methods*

The analysis was divided into three groups: Correlation between tumor tissue variables (four variables, yielding six pairs), correlation between tumor tissue variables and imaging data (four times three imaging variables, yielding 12 pairs for the baseline analysis, as well as another four variables times two delta variables, yielding eight pairs for the development of hypoxia during treatment), and finally influence of tumor tissue variables on outcome parameters (namely overall survival and recurrence-free survival). For the first two groups, Pearson's product-moment correlation was performed to analyze the relation between the pairs of markers or parameters, respectively. For the third group, the Cox proportional hazards model was used.

## Results

Sixteen patients were included in this project. Initial hypoxic subvolumes on the pretreatment 18F-MISO-PET scans varied from 1 to 73.8 ml with a mean of 15.8 ml and a median of 11.3 ml. The initial ratio of SUVmax in the tumor to SUVmean in the muscle varied from 1.14 to 2.41 with a mean of 1.9 and a median of 2.0 (see Table 1).

The H-scores resulting from the immunohistochemical analyses of CAIX, Ku80 and CD44 varied for the three different markers from 5 to 130, 120 to 280 and 250 to 300, respectively (see Table 1). The assessment of HPV status via LCD-chip analysis showed that only two patients were HPV-positive (also Table 1). Evaluating our staining for CAIX, we also observed a focal or patchy positivity with expression being accentuated in surface areas (see Figure 1).

Pairwise correlation analysis between each of the H-scores for Ku80, CD44 and CAIX and between any of these H-scores and each of the imaging variables showed no statistically significant correlation (see Table 2). A statistically significant negative correlation was found between CD44 H-score and HPV status ($r^2 = -.68$, $p = .004$). This result also remained statistically significant after Bonferroni-Holm adjustment for multiple testing. We also analyzed the correlation between the above-mentioned H-scores and the delta of the normalized SUVmax tu values between the first and the second, as well as the first and the third 18F-MISO-PET scan. However, there were no statistically significant correlations between the changes in hypoxia during treatment and the baseline tissue-based markers (details not shown). In addition to these correlation analysis, Cox regression analysis was performed to analyze the influence of the tissue-based biomarkers on overall survival and recurrence-free survival (see Table 3). Due to the limited number of patients with HPV-positivity (2 vs. 14), this parameter could not be included in the regression analysis. One statistically significant factor was found: CAIX, with

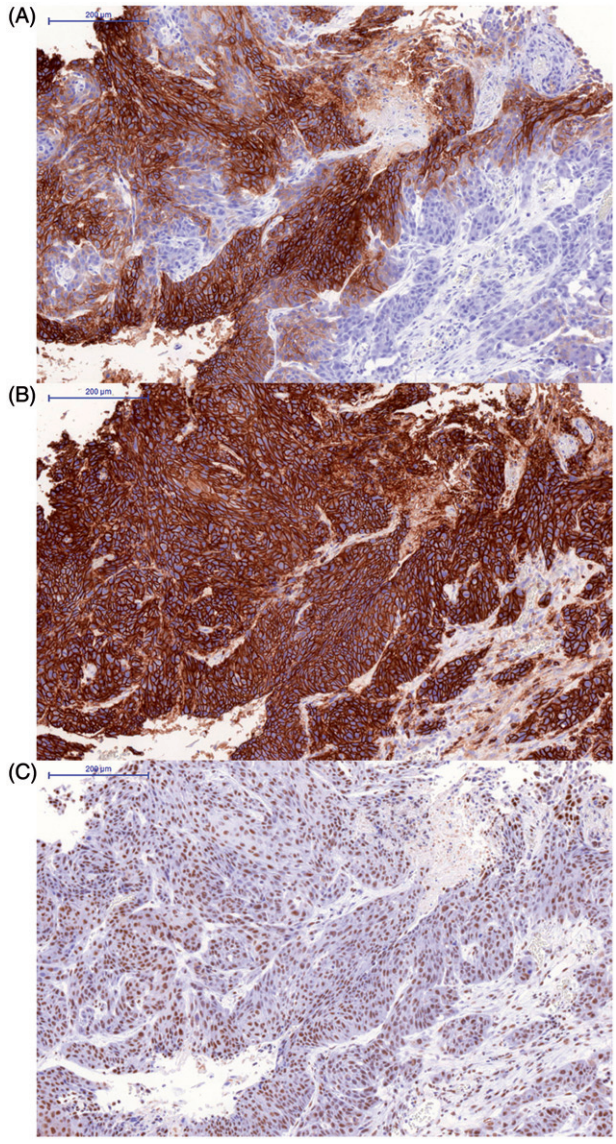


**Figure 1.** Immunohistochemical staining for CAIX (a), CD44 (b) and Ku80 (c). Of note the focal positivity for CAIX within the carcinoma (Objective magnification 10×).

a hazard ratio per unit of 0.96 in overall survival (95% confidence interval 0.93–1.00) at a p-value of 0.047. This is also exemplified in a Kaplan-Meier curve for overall survival, when dichotomizing at the median of CAIX (Figure 2). Of note, this is not a direct representation of the Cox proportional hazards model, but rather a different way of graphically representing the underlying result with regard to the influence of CAIX on survival for illustrative purposes, and is linked with a loss of test power, leading to a non-significant log-rank test of 0.347.

## Discussion

Evidence on the correlation between hypoxia and metabolic imaging, clinical outcome and the expression of surface markers in immunohistochemistry is scarce. We selected a small array of well known and recently investigated markers and compared their expression in immunohistochemical/array techniques with PET and clinical data.

**Table 2.** Pearson's product-moment correlation between tumor tissue variables and between these variables and imaging.

| | $r^2$ | p-value (* = significant, also after Bonferroni-Holm adjustment) |
|---|---|---|
| Correlation between tumor tissue variables (4 variables, 6 pairs) | | |
| CD44 – Ku80 | .13 | .62 |
| CD44 – CAIX | .10 | .72 |
| CD44 – HPV chip positive | −.68 | .0038* |
| Ku80 – CAIX | −.09 | .75 |
| Ku80 – HPV chip positive | −.25 | .34 |
| CAIX – HPV chip positive | −.083 | .78 |
| Correlation between tumor tissue variables and imaging (4 times 3 variables, 12 pairs) | | |
| CD44 – Volume FDG | .16 | .55 |
| CD44 – Volume MISO | −.02 | .95 |
| CD44 – MISO normalized SUVmax tu | .18 | .49 |
| Ku80 – Volume FDG | .23 | .39 |
| Ku80 – Volume MISO | .23 | .40 |
| Ku80 – MISO normalized SUVmax tu | .10 | .72 |
| CAIX – Volume FDG | .18 | .51 |
| CAIX – Volume MISO | .21 | .43 |
| CAIX – MISO normalized SUVmax tu | .04 | .88 |
| HPV chip positive – Volume FDG | −.08 | .78 |
| HPV chip positive – Volume MISO | −.02 | .96 |
| HPV chip positive – MISO normalized SUVmax tu | −.11 | .68 |

**Table 3.** Cox proportional hazards model.

| Tumor tissue variables and overall survival | | |
|---|---|---|
| Cox model | Hazard ratio per unit (95% confidence interval) | p-Value |
| CD44 | 1.07 (0.99–1.14) | .08 |
| Ku80 | 1.02 (1.00–1.05) | .07 |
| CAIX | 0.96 (0.93–1.00) | .047* |
| **Tumor tissue variables and recurrence-free survival** | | |
| Cox model | Hazard ratio per unit (95% confidence interval) | p-Value |
| CD44 | 1.03 (0.98–1.09) | .21 |
| Ku80 | 1.01 (0.99–1.03) | .18 |
| CAIX | 0.98 (0.96–1.01) | .14 |

*: significant.

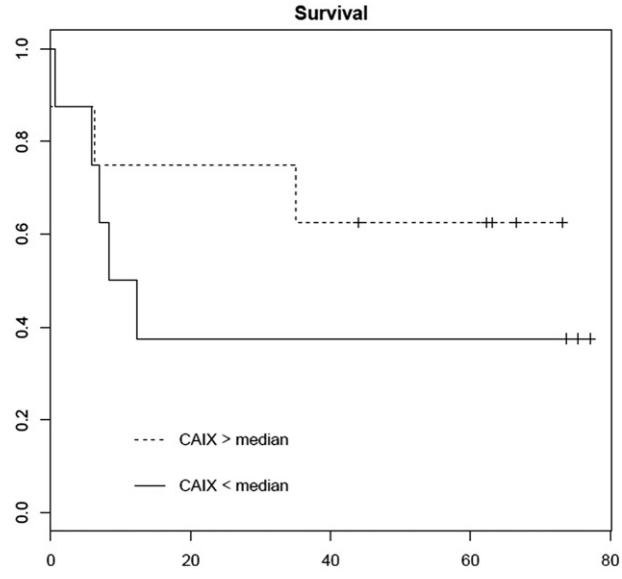


**Figure 2.** Kaplan-Meier curve for overall survival in relation to CAIX status (dichotomized at the median).

The development of the hypoxic imaging in the course of treatment – assessed by serial imaging – and also the relation of SUV values in the tumor and hypoxic subvolumes with regard to clinical outcome are subject to a dedicated and more detailed analysis [13]. In short, it has been demonstrated that all patients with residual hypoxia showed local recurrence, whereas none with completely resolving hypoxia did. However, due to tumor biopsies only taken once before commencing treatment, it was the primary goal of this research project to correlate the information available from the tumor biopsies with those obtainable with the closest temporal proximity (i.e. the first pretreatment imaging time point). As a secondary goal, we also analyzed whether we could establish a correlation between the baseline tissue-based biomarkers and the development of hypoxia during treatment, as assessed via serial 18F-MISO-PET imaging. The underlying hypothesis was whether the pretreatment expression of certain markers could be predictive of the development of hypoxia during therapy, and might therefore serve as a potential treatment stratifier.

CAIX is an endogenous marker of hypoxia. It has been described to be associated with reduced survival in head and neck and oral cavity tumor patients [17]. To our best knowledge, there are no data correlating CAIX and hypoxia imaging in humans. In a small study with DCE-MRI and H-MRS, CAIX was not significantly correlated with these imaging data [18]. In a large patient cohort, CAIX was significantly associated with cancer-specific and overall survival; however, there was no significant correlation between CAIX and $pO_2$-polarography [19]. There is a recent study however, finding a weak correlation between HIF-1alpha and 18F-MISO-PET [14], which has been published after completion of this study. Earlier, others have shown that hypoxic volumes delineated with 18F-MISO-PET show high correlations with both CAIX and Pimonidazole staining using immunohistochemistry in a rat model [20]. However, in their study the whole tumor was excised and microscopically analyzed, thus preventing the possibility of missing the hypoxic region, as given in the biopsies taken from the human trial subjects in our study. We found no correlation between CAIX and 18F-MISO-PET imaging, however, we did see a statistically significant reduction of hazard ratio in overall survival ($p = .047$). This finding is surprising, yet it is difficult to interpret given the unclear question of representability of the area of the tumor subject to immunohistochemical analysis. Interestingly, Rademakers et al. also found a low CAIX expression in biopsies to be an independent prognostic factor associated with worse outcome in patients with head and neck cancer undergoing radiotherapy. They comment on the low correlation between CAIX and other forms of hypoxia measurement, and instead of CAIX fraction, they find the staining pattern to be more informative, with a focal, peri-necrotic staining being strongly indicative of reduced local control, metastasis-free survival and overall survival [21]. We also observed a focal positivity in our samples. However, as quantification following the H-score method is of higher usability for routine practice, we did not further investigate specific staining patterns. In addition, in our experience they also suffer from a high inter-observer variability and therefore often deliver results which are difficult to reproduce.

HPV infection has been shown to be a significant factor regarding patient survival [22]. A recent study analyzed the relation between HPV status and hypoxia, assessed using pretreatment 18F-MISO-PET; the results in 63 patients with head and neck cancer showed that both HPV-positive and -negative tumors have a high prevalence of hypoxia. As expected, the outcome was worst for patients with HPV-negative hypoxic tumors [15]. In our study, due to the small number of HPV-positive cases (2 vs. 14), no definite conclusions could be drawn in this regard. This fact also explains why it is difficult to interpret the statistically significant negative correlation between CD44 H-score and HPV status ($p = .004$). However, HPV – as a positive prognostic factor – could also be a surrogate marker for stem cell enrichment within the tumor, with a higher stem cell enrichment also explaining worse prognosis (then again indicated by an increase in visibility of CD44).

Ku80 is a protein involved in non-homologous end-joining, a part of DNA double-strandbreak repair, which has recently been found to be associated with locoregional failure and increased mortality in head and neck tumors, especially when HPV-negative [23]. The fourth tissue-based biomarker investigated in this study, CD44, is a putative stem cell marker which has been correlated with local recurrence in laryngeal carcinoma [24]. However, our analysis revealed no statistically significant correlation between the markers mentioned above and the PET or clinical data. This was also true for the delta between the first and the second and the third 18F-MISO scans performed during radiochemotherapy in weeks 2 and 5. It can be argued, however, that we might be detecting a trend which is not significant due to the restricted sample size. In the case of overall survival, an increase in CD44 and Ku80 seems to indicate a worse prognosis ($p = .08$ and $p = .07$, respectively). In both cases, this link could be seen as biologically plausible, taking into consideration the above-mentioned studies of Moeller and de Jong [23,24].

Taken together, the tissue-based biomarkers included in this study are not correlated with one another (with the unclear exception of CD44 and HPV), with the imaging variables and/or the outcome (with the exception of CAIX).

Some hypotheses regarding the majority of negative results regarding the correlation with imaging and the influence on survival will be presented here:

First, the markers used in this study were only scored in one small biopsy obtained to confirm diagnosis of malignancy. Therefore, we were not able to account for intratumor heterogeneity, which has been identified as a major characteristic of solid tumors [25].

Second, we only examined a relatively small patient number. This of course results in reduced statistical power and did not allow for more sophisticated statistical analysis. However, given that hypoxia imaging is still exclusively used for research purposes and only available in a limited number of centers, the number of patients undergoing 18F-MISO-PET imaging is generally quite small. A potential solution to this issue is establishing multi-center data collection and

combined analysis. The German Cancer Consortium is currently setting up a respective registry addressing these issues. The data to be collected there will offer interesting possibilities in the future.

In summary, when comparing the evidence provided by other hypoxia imaging studies and the results of this study, it can be stated, that biological imaging is capable of independently predicting response, and thus adds value to the clinical setting. This has not been seen for the tissue-based biomarkers employed in this study. Also taking into account the difficulties in analyzing markers obtained from a single biopsy, it can be hypothesized, that PET imaging may offer several advantages over information gained from single small biopsies despite its lower resolution: Biological imaging integrates information from the whole tumor and adjacent lymph nodes, in contrast to a single biopsy, which only assesses a fraction of tumor mass [26]; it is non-invasive and can therefore more easily be done serially; finally, in the future, it may offer a possibility to stratify patients to receive different forms of treatment [8].

## Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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