



**Analysis of relation between hypoxia imaging using 18F-MISO-PET and tissue-based biomarkers in patients with head and neck tumours in the course of primary radiochemotherapy**

Journal:	<i>Acta Oncologica</i>
Manuscript ID	SONC-2016-0190
Manuscript Type:	Original Manuscript
Date Submitted by the Author:	29-Mar-2016
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Keywords:	tumour hypoxia, hypoxia imaging, biological imaging, head and neck cancer, immunohistochemistry

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10 **Analysis of relation between hypoxia imaging using 18F-MISO-PET and tissue-based**  
11 **biomarkers in patients with head and neck tumours in the course of primary**  
12 **radiochemotherapy**  
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**RUNNING TITLE**

Hypoxia imaging and biomarkers in head and neck cancer

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**TITLE:**

**Analysis of relation between hypoxia imaging using 18F-MISO-PET and tissue-based biomarkers in patients with head and neck tumours in the course of primary radiochemotherapy**

**ABSTRACT:**

**Background:** Tumour hypoxia is associated with poor prognosis and outcome and can be visualized using 18F-MISO-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 CT. Tumour biopsies were stained for three tissue-based markers (Ku80, CAIX, CD44); in addition, HPV status was assessed. H-scores of marker expression were generated and the results were correlated with the biological imaging and clinical outcome.

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6 **Results:** No statistically significant correlation was established between the H-scores for Ku80,  
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8 CD44 and CAIX or between any of the H-scores and the imaging variables (tumour volume on  
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10 18F-FDG-PET in ml, hypoxic subvolume as assessed by 18F-MISO-PET in ml, and SUVmax  
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12 tumour / SUVmean muscle during the 18F-MISO-PET). A statistically significant negative  
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14 correlation was found between CD44 H-score and HPV status ( $p=0.004$ ). Cox regression analysis  
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16 for overall survival and recurrence-free survival showed one significant result for CAIX being  
17  
18 associated with improved overall survival (Hazard ratio 0.96 (0.93-1.00),  $p=0.047$ ).  
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22 **Conclusion:** Expression of Ku80, CAIX and CD44 as assessed by immunohistochemistry of  
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24 tumour biopsies were not correlated to one another or the biological imaging data. However,  
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26 there was a significant influence of CAIX on overall survival and between CD44 and HPV.  
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50 **KEYWORDS:**

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53 tumour hypoxia; hypoxia imaging; biological imaging; head and neck cancer;  
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## INTRODUCTION

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 tumour 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 tumour 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, <sup>18</sup>F-MISO-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<sub>2</sub>-polarography measurements in head and neck tumours 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 <sup>18</sup>F-MISO-PET scans for tumour hypoxia imaging in addition to <sup>18</sup>F-FDG-PET [3][13].

On the other hand, the relation between tissue-based biomarkers and the information

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6 gathered from biological imaging are not well understood and there are only few studies  
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8 addressing this issue.  
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11 The goal of this study is to evaluate the correlation between select tissue-based biological  
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13 markers and data obtained from PET imaging as well as outcome parameters in the same group  
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15 of patients, and thus to elucidate the relation between  
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19 1. The expression of the selected tissue-based biomarkers and HPV status themselves.
- 20  
21 2. The expression of tissue-based biomarkers and hypoxia & metabolic imaging.
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23 3. The expression of tissue-based biomarkers and clinical outcome.  
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29 As tissue-based biomarkers, we chose carbonic anhydrase IX (CAIX) as cellular correlate for  
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31 tumour hypoxia, CD44 as putative stem cell marker, Ku80 which is involved in DNA double-  
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33 strand-break repair and human papilloma virus (HPV), another well-known prognostic factor in  
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35 head and neck cancer patients.  
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39 The information gathered from these investigations was hypothesized to guide the use of the  
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41 afore-mentioned tissue-based biomarkers in relation to the use of hypoxia and metabolic  
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43 imaging.  
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## MATERIAL AND METHODS

### Cohort

16 patients with HNSCC were included in this study. A detailed characterisation of the study population is given elsewhere [13]. However, this study reports a cohort with an updated and prolonged follow-up. The primary tumour localisations (patient numbers in brackets) were oral cavity (1), oropharynx (7), hypopharynx (5) and larynx (3). The patients were treated with primary radiochemotherapy; the protocol consisted of a total dose of 70 Gy in five fractions per week over 7 weeks, and up to three concomitant cycles of cisplatin (weeks 1, 4 and 7). The protocols for the imaging study and the subsequent tumour 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 tumour sample and correlation analysis.

### Imaging and radiotherapy

All patients received an 18F-FDG-PET scan, a planning CT and MRI before starting the treatment. Up to three 18F-MISO-PET scans were performed: before starting the treatment and in weeks two and five. For this analysis, only the pre-treatment scan has been incorporated into the analysis because of the closest temporal proximity to the tumour biopsy. Scans were done with Siemens ECAT EXACT 921 PET scanner (15 patients) and Philips Gemini TrueFlight PET/CT scanner (1 patient). The dose of 18F-MISO was 3.7 MBq/kg up to a maximum of 370 MBq; image acquisition started 150 min after tracer injection. Scan duration was 35 min (3

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6 frames at 10 min, followed by a 5 min transmission scan). Patients were immobilized identical  
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8 to the radiation position with a head and neck mask. Having acquired the 18F-MISO-PET scans,  
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10 these were co-registered with the CT scans (for the stand-alone ECAT scanner).  
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14 Gross tumour volume (GTV) was defined according to the 18F-FDG-PET scan; an uptake  
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16 exceeding 40% of the maximum standardized uptake value was set as threshold and the  
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18 resulting volume was validated with the CT and MRI data by an experienced clinician judging  
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20 clinically acceptable quality of co-registration and image alignment as well as accuracy of the  
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22 volume delineation. Hypoxic volumes were defined as all voxels within the GTV with a ratio of  
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24 standardized uptake value of the tumour to mean standardized uptake value in a contralateral  
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26 normal tissue sphere of equal to or greater than 1.4 (normalized against blood activity  
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28 measured in the left ventricle).  
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### 33 **Immunohistochemistry**

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36 Tumour biopsies were taken by head and neck surgeons four to six weeks before initiation of  
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38 treatment to confirm diagnosis. Tumour biopsies were analysed for marker expression and  
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40 correlation analyses were performed. All tumour samples were formalin fixed and paraffin-  
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42 embedded using routine protocols. 2 µm sections of tumour biopsies were mounted on coated  
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44 glass slide (Superfrost Plus, Langenbrinck) deparaffinised and rehydrated using a descending  
45  
46 alcohol row. Heat induced antigen retrieval was performed using citrate buffer at pH 6.1 for 40  
47  
48 minutes. After inhibition of endogenous peroxidase by H<sub>2</sub>O<sub>2</sub> (5 minutes) primary antibodies  
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50 (CAIX: Cell Signalling, monoclonal rabbit anti-human 1:100; CD44: Cell Signalling, monoclonal  
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52 mouse anti-human 1:500; Ku 80: Cell Signalling rabbit anti-human, 1:300) were incubated for  
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54 30 minutes. Primary antibody detection and visualization was performed with the DAKO FLEX  
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6 EnVision Kit using the rabbit linker (CAIX and Ku 80) and mouse linker (CD44) for 15 minutes  
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8 followed by horseradish peroxidase (20 minutes) and diaminobenzidine (10 minutes). All slides  
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10 were counterstained by hematoxilin, dehydrated in an ascending alcohol row and coverslipped.  
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14 For microscopic analysis, all slides were digitized using a Panoramic Scan (3D Histech, Hungary).  
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16 Visual assessment was performed using an H-score, thereby taking into account the respective  
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18 percentages of regions with strong, moderate and weak staining and yielding a final score of 0  
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20 to 300, as previously described [14].  
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### 22 23 24 **HPV-Analysis**

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27 After microdissection of tumour tissue from marked histological slides, DNA was isolated using  
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29 routine protocols. For detection of HPV specific DNA the Chipron HPV 3.5 LCD array kit (Chipron  
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31 GmbH, Berlin, Germany) was used according to the supplier's instructions, yielding a binary +/-  
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33 score. Positive and negative controls were performed for each run.  
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### 36 37 **Statistical methods**

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40 The analysis was divided into three groups: Correlation between tumour tissue variables (4  
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42 variables, yielding 6 pairs), correlation between tumour tissue variables and imaging data (4  
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44 times 3, yielding 12 pairs), and finally influence of tumour tissue variables on outcome  
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46 parameters (namely overall survival and recurrence-free survival). For the first two groups,  
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48 Pearson's product-moment correlation was performed to analyse the relation between the  
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50 pairs of markers or parameters, respectively. For the third group, the Cox proportional Hazards  
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52 model was used.  
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## RESULTS

16 patients were included in this project. Initial hypoxic tumour subvolume on the pre-treatment 18F-MISO-PET scan 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 tumour to SUVmean in the muscle varied from 1.14 to 2.41 with a mean of 1.9 and a median of 2.0.

The development of the hypoxic imaging in the course of treatment – assessed by serial imaging – and also the relation of SUVmax and hypoxic subvolume in regard to clinical outcome are subject to a dedicated and more detailed analysis [13]. In short, it has been shown that all patients with residual hypoxia showed local recurrence, whereas none with completely resolving hypoxia did. However, due to tumour biopsies only taken once before commencing treatment, it was the goal of this research project to correlate the information available from the tumour biopsies with those obtainable with the closest temporal proximity (i.e. the first, pre-treatment imaging time point).

Immunohistochemistry was performed for CAIX, Ku80 and CD44. Representative examples of the staining are given in FIGURE 1. In addition, HPV status was investigated via LCD-chip analysis. The immunohistochemistry staining was scored and a cumulative H-score calculated. These results are presented in detail in TABLE 1, combined with the outcome data and imaging parameters.

The details of the results of the statistical analysis with regard to correlation between the tissue-based biomarkers and the biological imaging are given in TABLE 2. 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. The



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6 imaging variables used for the analysis were the tumour volume on 18F-FDG-PET measured in  
7 ml, the hypoxic subvolumes as assessed by the 18F-MISO-PET measured in ml, and the ratio of  
8 SUVmax in the tumour and SUVmean in muscle during the 18F-MISO-PET scan. A statistically  
9 significant negative correlation was found between CD44 H-score and HPV status ( $r^2 = -0.68$ ,  $p =$   
10 0.004). This result also remained statistically significant after Bonferroni-Holm adjustment for  
11 multiple testing. In addition to these correlation analysis, Cox regression analysis was  
12 performed to analyse the influence of the tissue-based biomarkers on overall survival and  
13 recurrence-free survival. These results are given in TABLE 3. Due to the limited number of  
14 patients with HPV-positivity (2 vs. 14), this parameter could not be included in the regression  
15 analysis. One statistically significant factor was found: CAIX, with a hazard ratio per unit of 0.96  
16 in overall survival (95% confidence interval 0.93-1.00) at a p-value of 0.047. This is also  
17 exemplified in FIGURE 2, where we show a Kaplan-Meier-curve for overall survival, when  
18 dichotomising at the median of CAIX. Of note, this is not a direct representation of the Cox  
19 proportional hazards model, but rather a different way of graphically representing the  
20 underlying result with regard to the influence of CAIX on survival.  
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## 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.

CAIX is an endogenous marker of hypoxia. It has been described to be associated with reduced survival in head and neck and oral cavity tumour patients [15]. 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 [16]. 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<sub>2</sub>-polarography [17]. There is a recent study however, finding a weak correlation between HIF-1alpha and 18F-MISO-PET [18], 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 [19]. However, in their study the whole tumour was excised and microscopically analysed, 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 = 0.047). This finding is surprising, yet it is difficult to interpret given the unclear question of representability of the area of the tumour subject to immunohistochemical analysis.

Human papilloma virus (HPV) infection has been shown to be a significant factor regarding

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6 patient survival [20]. A recent study analysed the relation between HPV status and hypoxia,  
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8 assessed using pre-treatment 18F-MISO-PET; the results in 63 patients with head and neck  
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10 cancer showed that both HPV positive and negative tumours have a high prevalence of  
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12 hypoxia. As expected, the outcome was worst for patients with HPV negative hypoxic tumours  
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14 [21]. In our study, due to the small number of HPV-positive cases (2 vs. 14), no definite  
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16 conclusions could be drawn in this regard. This fact also explains why it is difficult to interpret  
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18 the statistically significant negative correlation between CD44 H-score and HPV status ( $p =$   
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20 0.004). On the other hand, HPV – as a positive prognostic factor – could also be a surrogate  
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22 marker for stem cell enrichment within the tumour, with a higher stem cell enrichment also  
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24 explaining worse prognosis (then again indicated by an increase in visibility of CD44).  
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30 Ku80 is a protein involved in non-homologous end-joining, a part of DNA double-strand-break  
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32 repair, which has recently been found to be associated with locoregional failure and increased  
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34 mortality in head and neck tumours, especially when HPV-negative [22]. The fourth tissue-  
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36 based biomarker investigated in this study, CD44, is a putative stem-cell marker which has been  
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38 correlated with local recurrence in laryngeal carcinoma [23]. However, our analysis revealed no  
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40 statistically significant correlation between the markers mentioned above and the PET or  
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42 clinical data. It be argues, however, that our statistical analysis is detecting a trend which is not  
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44 significant due to the restricted sample size. In the case of overall survival, an increase in CD44  
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46 and Ku80 seems to indicate a worse prognosis ( $p = 0.08$  and  $p = 0.07$ , respectively). In both  
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48 cases, this link could be seen as biologically plausible, taking into consideration the above-  
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50 mentioned studies of Moeller and de Jong [22][23].  
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56 In summary, the tissue-based biomarkers included in this study are not correlated with one  
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58 another (with the unclear exception of CD44 and HPV), with the imaging variables and/or the  
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6 outcome (again, with the surprising exception of CAIX).  
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10 Some hypotheses regarding the majority of negative results regarding the correlation with  
11 imaging and the influence on survival will be presented here:  
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14 First, the markers used in this study were only scored in one small biopsy obtained to confirm  
15 diagnosis of malignancy. Therefore, we were not able to account for intra-tumour  
16 heterogeneity, which has been identified as a major characteristic of solid tumours [24].  
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20 Second, we only examined a relatively small patient number. This of course results in reduced  
21 statistical power and did not allow for more sophisticated statistical analysis. However, given  
22 that hypoxia imaging is still exclusively used for research purposes and only available in a  
23 limited number of centres, the number of patients undergoing 18F-MISO-PET imaging is  
24 generally quite small. A potential solution to this issue is establishing multi-centre data  
25 collection and combined analysis. The German Cancer Consortium (DKTK) is currently setting  
26 up a respective registry addressing these issues. The data to be collected there will offer  
27 interesting possibilities in the future.  
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31 In summary, when comparing the evidence provided by other hypoxia imaging studies and the  
32 results of this study, it can be stated, that biological imaging is capable of independently  
33 predicting response, and thus adds value to the clinical setting. This has not been seen for the  
34 tissue-based biomarkers employed in this study. Also taking into account the difficulties in  
35 analysing markers obtained from a single biopsy, it can be hypothesized, that PET imaging may  
36 offer several advantages over information gained from single small biopsies despite its lower  
37 resolution: Biological imaging provides an comprehensive information from the tumour and  
38 adjacent lymph nodes, in contrast to a small sample biopsy, which only assesses a fraction of  
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6 tumour mass [25]; it is non-invasive and can therefore more easily be done serially; finally, in  
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8 the future, it may offer a possibility to stratify patients to receive different forms of treatment  
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11 [8].  
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## 13 14 15 16 17 **CONCLUSION**

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20 With this study we correlated biological imaging using 18F-MISO-PET and 18F-FDG-PET as well  
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22 as clinical outcome data with immunohistochemistry and chip array data obtained from 16  
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24 patients with head and neck tumours. No statistically significant, clinically directly relevant  
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26 correlations were revealed. The findings of this exploratory study underline the need for large,  
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28 prospective data collections, such as those put forward by the German Cancer Consortium  
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30 (DKTK).  
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## 34 35 **ACKNOWLEDGEMENTS**

36  
37 This work was supported by the German Consortium for Translational Cancer Research  
38  
39 (Deutsches Konsortium für Translationale Krebsforschung, DKTK), partner site Freiburg, and a  
40  
41 research grant of the Mueller-Fahnenberg-Foundation, Freiburg. The funders had no  
42  
43 involvement in study design, collection, analysis, and interpretation of data, writing of the  
44  
45 report, or in the decision to submit the article for publication.  
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## 50 **CONFLICT OF INTEREST**

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53 None declared.  
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**Table 1:** Patient characteristics, imaging data (pre-treatment 18F-FDG-PET and 18F-MISO-PET) and cumulative H-score for immunohistochemistry staining for CAIX, Ku80 and CD44 as well as results of HPV Chip analysis for patients 1 to 16

Patient- No.	Age	Gender	Location	TNM	Follow- up (months)	Local failure	Death	Volume FDG (ml)	Volume MISO (ml)	MISO SUVmax tumour / SUVmean muscle	CD44 - H- score	Ku80 - H- score	CAIX - H- score	HPV- Chip positive
1	64	M	oropharynx	T4 N2b	77.1			26	7.4	2.2	300	180	15	0
2	52	M	oropharynx	T4 N2c	7.3	recurrence	tumour	20	14.9	2.4	300	210	5	0
3	61	F	oral cavity	T4 N2c	0	no remission	tumour	99	73.8	2.4	280	260	25	0
4	75	F	tonsil	T2 N2c	0.6		other	24	4.7	1.6	280	240	20	0
5	62	M	larynx	T4 N2c	35.1		second malignancy	10	1.9	1.5	285	230	30	0
6	48	M	hypopharynx	T4 N2c	6.3	recurrence	tumour	29	17.2	2.4	300	270	45	0
7	68	F	base of tongue	T3 N2c	73.7			11	10.3	1.7	250	270	0	1
8	47	M	oropharynx	T4 N2c	12.3		metastasis	20	15	1.6	280	170	10	0
9	71	M	larynx	T4 N2c	62.3			16	12.2	1.7	270	200	90	0
10	69	M	larynx	T3 N2c	75.3			9	5.3	2.3	280	210	5	0
11	66	M	hypopharynx	T3 N2c	44	recurrence		16	15.8	2.3	280	200	130	0
12	51	M	hypopharynx	T3 N2c	5.9		other	25	2.9	1.5	295	240	5	0
13	44	M	hypopharynx	T4 N2b	73.1			22	1	1.1	300	220	75	0
14	61	M	tonsil	T4 N2c	66.5			35	19.8	1.9	270	120	65	1
15	51	M	tonsil	T4 N2c	63.1			62	42	2.2	300	280	120	0
16	44	M	hypopharynx	T4 N2c	8.3	recurrence	tumour	15	8	2.1	285	280	15	0

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

	$r^2$	df	p-value (* = significant, also after Bonferroni-Holm adjustment)
Correlation between tumour tissue variables (4 variables => 6 pairs)			
CD44 – Ku80	0.13	14	0.62
CD44 – CAIX	0.10	14	0.72
CD44 – HPV chip positive	-0.68	14	0.0038 *
Ku80 – CAIX	-0.09	14	0.75
Ku80 – HPV chip positive	-0.25	14	0.34
CAIX – HPV chip positive	-0.083	14	0.78
Correlation between tumour tissue variables and imaging (4 times 3 = 12 pairs)			
CD44 – Volume FDG	0.16	14	0.55
CD44 – Volume MISO	-0.02	14	0.95
CD44 – MISO SUVmax tu / SUVmean muscle	0.18	14	0.49
Ku80 – Volume FDG	0.23	14	0.39
Ku80 – Volume MISO	0.23	14	0.40
Ku80 – MISO SUVmax tu / SUVmean muscle	0.10	14	0.72
CAIX – Volume FDG	0.18	14	0.51
CAIX – Volume MISO	0.21	14	0.43
CAIX – MISO SUVmax tu / SUVmean muscle	0.04	14	0.88
HPV chip positive – Volume FDG	-0.08	14	0.78
HPV chip positive – Volume MISO	-0.02	14	0.96
HPV chip positive – MISO SUVmax tu / SUVmean muscle	-0.11	14	0.68

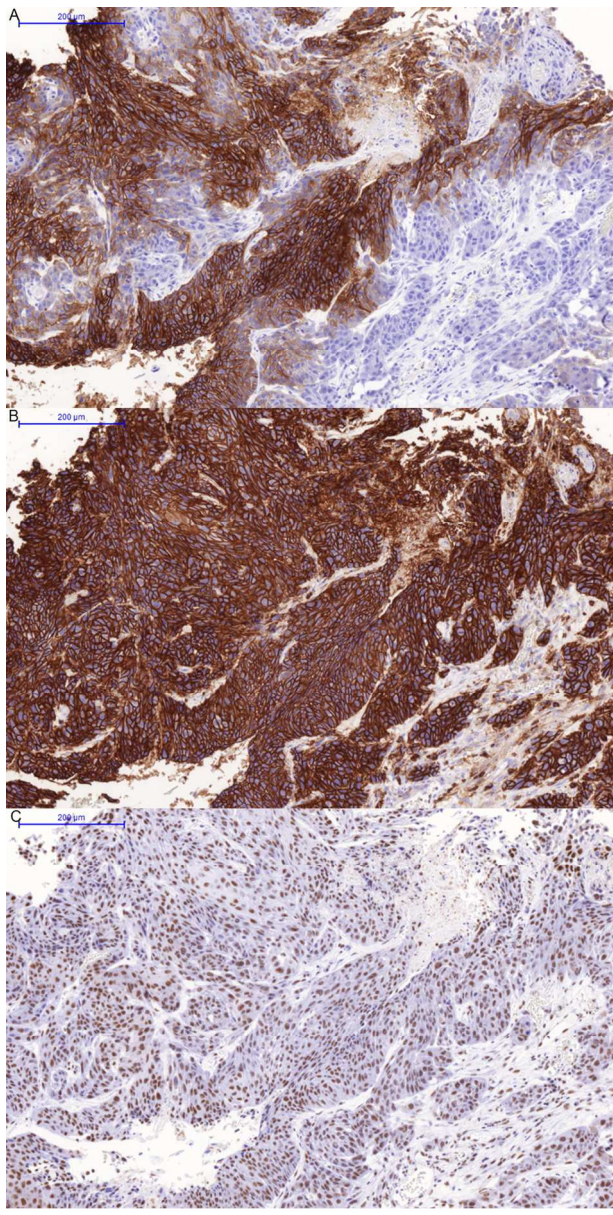
**Table 3:** Cox proportional hazards model

Tumour tissue variables and overall survival		
Cox model	Hazard ratio per unit (95% confidence interval)	p-value (* = significant)
CD44	1.07 (0.99 – 1.14)	0.08
Ku80	1.02 (1.00 – 1.05)	0.07
CAIX	0.96 (0.93 – 1.00)	0.047 *
Tumour tissue variables and recurrence-free survival		
Cox model	Hazard ratio per unit (95% confidence interval)	p-value
CD44	1.03 (0.98 – 1.09)	0.21
Ku80	1.01 (0.99 – 1.03)	0.18
CAIX	0.98 (0.96 – 1.01)	0.14

**Figure 1:** Immunohistochemical staining for CA IX (A), CD44 (B) and Ku80 (C). Of note the focal positivity for CA IX within the carcinoma (Objective magnification 10x).

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

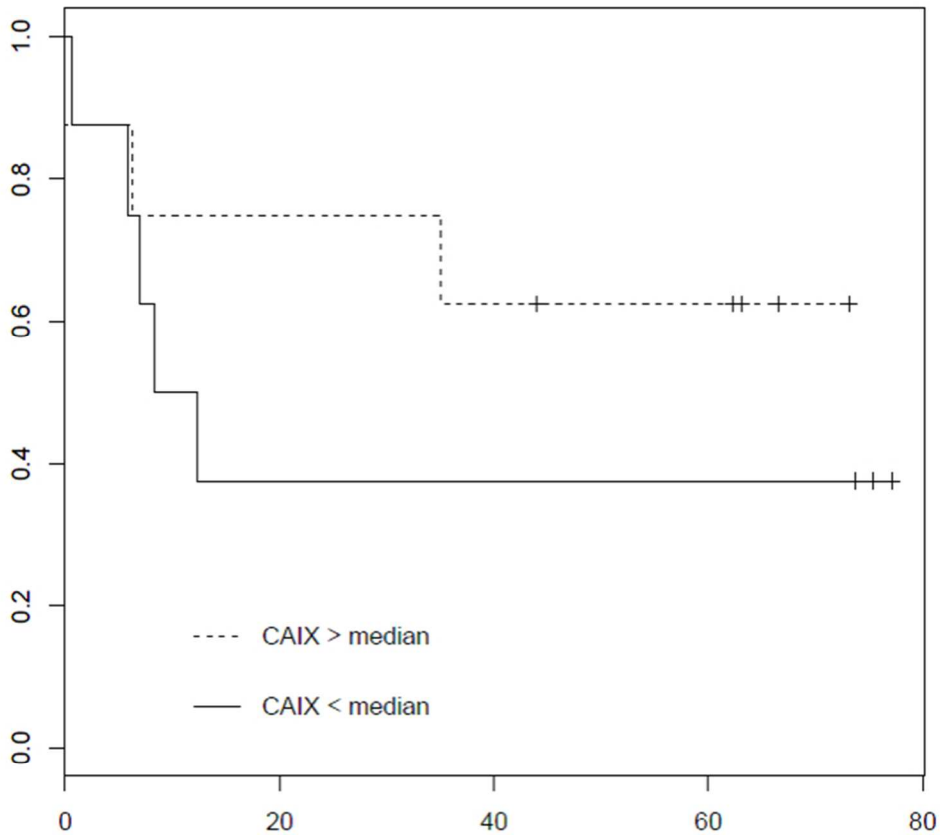
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### Survival



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