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Original Article

Repeat FMISO-PET imaging weakly correlates with hypoxia-associated gene expressions for locally advanced HNSCC treated by primary radiochemotherapy

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ABSTRACT

Background: Hypoxia is an important factor of tumour resistance to radiotherapy, chemotherapy and potentially immunotherapy. It can be measured e.g. by positron emission tomography (PET) imaging or hypoxia-associated gene expressions from tumour biopsies. Here we correlate [^{18}F]fluoromisonidazole (FMISO)-PET/CT imaging with hypoxia-associated gene expressions on a cohort of 50 head and neck squamous cell carcinoma (HNSCC) patients and compare their prognostic value for response to radiochemotherapy (RCTx).

Methods: FMISO-PET/CT images of 50 HNSCC patients were acquired at four time-points before and during RCTx. For 42 of these patients, hypoxia-associated gene expressions were evaluated by nanoString technology based on a biopsy obtained before any treatment. The FMISO-PET parameters tumour-to-background ratio and hypoxic volume were correlated to the expressions of 58 hypoxia-associated genes using the Spearman correlation coefficient ρ. Three hypoxia-associated gene signatures were compared regarding their correlation with the FMISO-PET parameters using their median expression. In addition, the correlation with tumour volume was analysed. The impact of both hypoxia measurement methods on loco-regional tumour control (LRC) and overall survival (OS) was assessed by Cox regression.

Results: The median expression of hypoxia-associated genes was weakly correlated to hypoxia measured by FMISO-PET imaging ($\rho \leq 0.43$), with higher correlations to imaging after weeks 1 and 2 of treatment ($p < 0.001$). Moderate correlations were obtained between FMISO-PET imaging and tumour volume ($\rho \leq 0.69$). Prognostic models for LRC and OS based on the FMISO-PET parameters could not be improved by including hypoxia classifiers.

Conclusion: We observed low correlations between hypoxia FMISO-PET parameters and expressions of hypoxia-associated genes. Since FMISO-PET showed a superior patient stratification, it may be the preferred biomarker over hypoxia-associated genes for stratifying patients with locally advanced HNSCC treated by primary RCTx.

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Tumour hypoxia is a well-known factor that adversely affects local tumour control and survival [1–5]. For head and neck squamous cell carcinomas (HNSCC), pre-treatment tumour oxygenation was shown to be an important prognostic factor for survival after primary radiotherapy applied alone or combined with chemotherapy, surgery, or radiation sensitizers [6–9]. Hypoxia modification has led to reduced incidences of local failures and distant metastases as well as to increased overall survival (OS) [10–13]. It has been argued that for hypoxic tumours dose escalation to the tumour or to its hypoxic sub-volumes may increase local control [14–18]. Since dose escalation may lead to more severe acute or late side effects, the tumour hypoxia status needs to be assessed using biomarkers, e.g. by hypoxia-associated gene signatures, immunohistochemical staining of tumour biopsies for hypoxia-related markers, blood biomarkers, or positron emission tomography (PET) using [^{18}F]fluoromisonidazole (FMISO), [^{18}F]fluoroazomycin-arabinoside (FAZA), or [^{18}F]flortanidazole (HX4) [7,19–30].

In our recently published prospective imaging study, repeat FMISO-PET imaging was used to determine tumour hypoxia at four timepoints prior to and during primary radiochemotherapy (RCTx) in 50 locally advanced HNSCC patients [19,23]. Residual hypoxia during radiochemotherapy was identified as a major driver of hypoxia-associated therapy resistance. Based on the FMISO-PET parameters TBR_{peak} and $HV_{1.6}$, patients at significantly increased risk of loco-regional recurrence were identified, which was successfully validated [23].

Molecular surrogates of tumour hypoxia can also be analysed on the transcriptional level [31–34]. In a recent study, we evaluated three previously described hypoxia-associated gene signatures on 158 patients with locally advanced HNSCC treated by primary RCTx within the German Cancer Consortium Radiation Oncology Group (DKTK-ROG) [35]. The study included the 15-gene signature developed by Toustrup et al. [31] based on hypoxia measurements in experimental studies, the 26-gene signature by Eustace et al. [32] including genes which relate to 10 well-known hypoxia-regulated genes, and the 30-gene signature by Lendahl et al. [33] that is based on an *in silico* meta-analysis of the NCBI Gene Expression Omnibus public microarray repository. In the subgroup of small tumours with a volume $\leq$19 cm^3, tumours classified as more hypoxic showed significantly lower loco-regional control (LRC) than less hypoxic tumours.

In this manuscript, we report on the correlation of FMISO-PET imaging to expressions of the 15-, 26- and 30-gene hypoxia-associated gene signature for the cohort of 50 patients presented in [19,23]. Moreover, the correlation of both hypoxia measures with tumour volume and their applicability for patient stratification regarding LRC and OS was analysed.

Patients and methods

Study design and patients

This study is a secondary post-project analysis on a cohort of 50 patients with locally advanced HNSCC, who were recruited between 2006 and 2013 in a prospective mono-centre single-arm non-randomised observational imaging trial that was registered publicly (www.clinicaltrials.gov, NCT00180180), and approved by the German Federal Radiation Protection Authority (Bundesamt für Strahlenschutz, Z5 – 22461/2 – 2004-061) and the local Ethics Committee (EK166082004). Inclusion and exclusion criteria were described previously [19]. In brief, inclusion criteria were: age $\geq$18 years, WHO performance status 0–2, histologically proven locally-advanced HNSCC, eligible for primary radiochemotherapy with curative intent. All patients gave written informed consent. Staging, treatment, imaging and follow-up have been described in detail [19,23]. Radiotherapy to a total dose of 72 Gy was combined with concurrent chemotherapy [19,36].

FMISO-PET/CT was performed before RCTx, at the end of the first and the second week of treatment and during the fifth week of RCTx. Molecular analyses were performed for 42 patients with sufficient tumour material using formalin-fixed paraffin-embedded (FFPE) tumour biopsies obtained at least 90 days before treatment. The median time between the acquisition of the biopsy and the first baseline FMISO-PET scan was 44 days (range: 18–79 days). The patient numbers available for correlation analyses are presented in Fig. 1 and the patients' characteristics of the 42 patients are presented in Table 1.

PET/CT imaging

The static FMISO-PET scans were acquired in 12 minutes per bed position on a Biograph 16 W PET/CT scanner (Siemens Medical Solutions Inc., Knoxville, TN) four hours after injection of 250–300 MBq FMISO [24,37]. After registration of PET and CT datasets, the gross tumour volume (GTV) was delineated on the pre-treatment CT taking into account clinical information as well as the FDG-positive volume. The mean standardised uptake value of FMISO-PET in a background region containing contralateral deep neck muscles (SUV_{mean_B}) was calculated. In addition, hypoxic volumes (HV) were defined encompassing voxels with SUVs larger than the SUV_{mean_B} multiplied by different factors, e.g. the factor 1.6 for $HV_{1.6}$ [20,28,38]. The peak tumour-to-background ratio (TBR_{peak}) was calculated as the SUV_{peak} (mean SUV within $5 \times 5 \times 5$ voxels (1.26 ml) of highest activity in the GTV) divided by the SUV_{mean_B}. Residual hypoxia after week 2 was defined as the ratio $HV_{1.6,week2}/HV_{1.6,baseline}$. Tumours were classified as more or less hypoxic based on cut-offs for the FMISO-PET parameters $HV_{1.6}$ and TBR_{peak} that were defined in the previous studies [19,23]. Further details on the imaging protocol, image registration and analysis are described elsewhere [19,23].

Analyses of hypoxia-associated gene signatures

For diagnosis, biopsies were taken from the peripheral edge of the clinically suspect primaries in the upper aero-digestive tract in order to histopathologically confirm squamous cell carcinoma. For gene expression analysis, one biopsy sample per patient was available. FFPE sections were first subjected to haematoxylin and eosin staining to histologically confirm the presence of squamous cell carcinoma. Afterwards, FFPE tissue specimens were subjected to RNA extractions using standardised procedures as described previously [35]. Gene expression analyses were performed using nanoString Elements technology (nanoString Technologies, Seattle, WA) [35]. The gene set contained 209 custom-selected genes that have previously been reported to be associated with sensitivity or resistance to radio(chemo)therapy. This included 58 hypoxia-associated genes contained in the previously developed 15-, 26- and 30-gene hypoxia-associated signatures [31–33], see Supplementary Table S1. Briefly, raw counts were logarithmised and then normalised to the mean of the internal level of reference genes *ACTR3*, *B2M*, *GNB2L1*, *NDFIP1*, *POLR2A*, *RPL11*, *RPL37A*, $x_n = \log_2(x_r) - \text{mean}_{REF}[\log_2(x_{r,REF})]$, where x_n is the normalised gene expression and x_r is the raw count of the considered gene, $x_{r,REF}$ represents the raw counts of the seven reference genes and the mean is calculated over these reference genes.

For the analysis of the individual hypoxia-gene signatures, the corresponding reference genes were used, see Supplementary Table S1. Tumours were classified as more or less hypoxic using k-mean clustering based on two cluster centres for every gene signature. In addition, the median expressions of the gene signatures were calculated after z-transforming the single gene expressions.

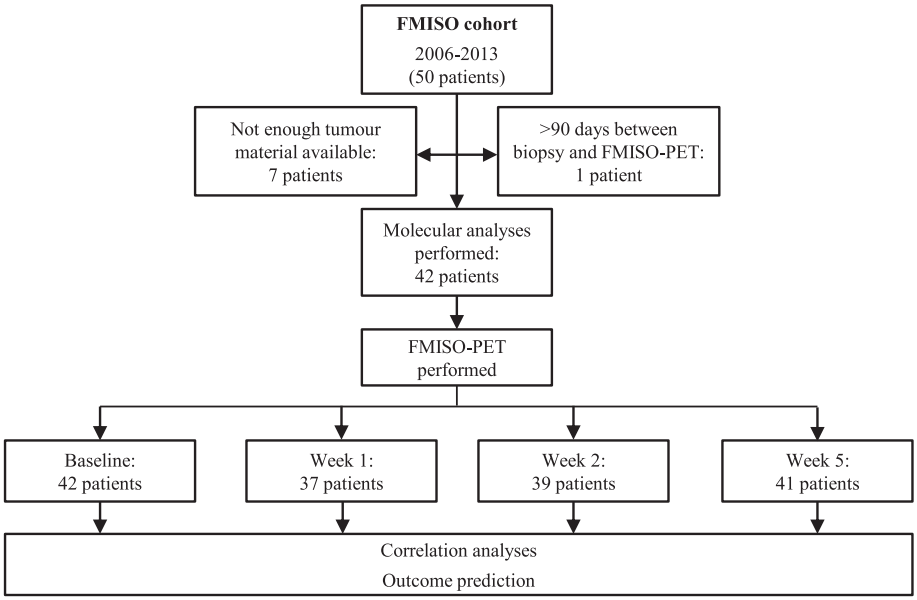

Fig. 1. Study design and patient numbers.

Table 1
Patient and tumour characteristics.

Characteristics	Median (range)	
Age (years)	55.5 (42–76)	
GTV (cm^3)	32.9 (5–178)	
Characteristics	Number of patients (of 42)	%
Gender		
Male	36	86
Female	6	14
Tumour localisation		
Oral cavity	10	24
Oropharynx	12	29
Hypopharynx	17	40
Larynx	3	7
Clinical tumour (T) stage		
cT3	13	31
cT4	29	69
Clinical nodal (N) stage		
cN0	6	14
cN1	4	9
cN2a	2	5
cN2b	7	17
cN2c	21	50
cN3	2	5
Clinical metastasis (M) stage		
cM0	42	100
Stage (UICC 7th edition)		
III	6	14
IV	36	86
Grading		
1	1	2
2	20	48
3	21	50
Smoking during therapy		
yes/no	31/11	74/26
Alcohol during therapy		
yes/no/unknown	20/19/3	48/45/7
HPV status		
p16-staining		
negative/positive	40/2	95/5
HPV-16-DNA		
negative/positive/unknown	33/4/5	79/9/12

Abbreviations: FU, follow-up; UICC, *Union Internationael Contre le Cancer*; GTV, gross tumour volume; HPV, human papilloma virus; p16, tumour suppressor gene encoded by the CDKN2A gene; DNA, deoxyribonucleic acid.

For this purpose, the genes *ATF7IP*, *HMMR*, *MYCBP*, *SLC7A1*, *SLC26A2* and *SOX4* with reported down-regulation under hypoxic conditions [33] were inverted before taking the median. Note that for the 30-gene signature, the gene *DHX34* was not available and the remaining 29 genes were analysed.

Statistical analyses

The primary endpoint was loco-regional tumour control (LRC) and the secondary endpoint was overall survival (OS). All endpoints were calculated from the first day of radiotherapy to the date of event or censoring. The impact of potential prognostic variables on the endpoints was evaluated using Cox regression. Correlations between FMISO-PET parameters and hypoxia-associated gene expressions were evaluated by the Spearman correlation coefficient ρ. Generalised linear regression was used to model the FMISO-PET parameters TBR_{peak} and $HV_{1.6}$ in dependence of the median expressions of the three gene signatures, GTV and their interaction. A Tweedie distribution with a logarithmic link function was applied since $HV_{1.6}$ was positively skewed and contained zero values. The natural logarithm (ln) of the GTV was considered in all analyses. Statistical analyses were performed using IBM SPSS Statistics 25 (IBM Corporation, Armonk, NY), two-sided tests were applied and p-values <0.05 were considered statistically significant. Due to the exploratory nature of this study and the limited sample size, multiple testing corrections were not applied.

Results

Spearman's correlations between the expressions of the 58 hypoxia-associated genes evaluated at baseline and the FMISO-PET parameters TBR_{peak} and $HV_{1.6}$ evaluated at baseline and after week 2 of treatment are depicted in Fig. 2. At baseline correlations ranged between −0.38 and 0.36 (median 0.02), while after week 2 correlations were significantly increased (range: −0.25 to 0.60, median 0.16, $p < 0.001$). The expressions of the five genes *ADM*, *EGLN3*, *ENO2*, *P4HA2* and *PGK1* showed a correlation larger than 0.4 to at least one of the FMISO-PET parameters after the second week of treatment, while no gene showed this moderate correlation to an FMISO-PET parameter at baseline. The highest correla-

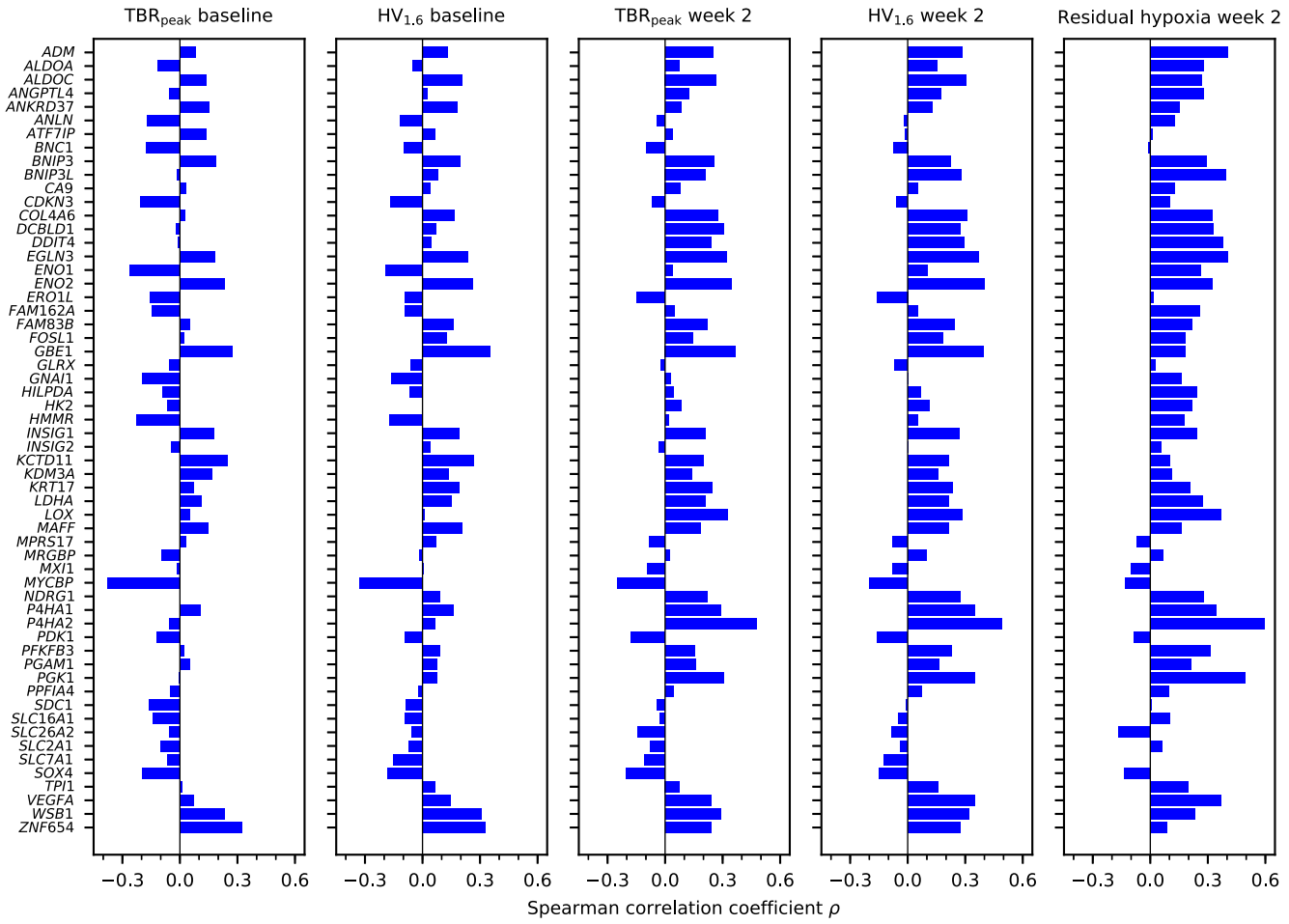

Fig. 2. Spearman's correlation coefficients ρ between the 58 hypoxia-associated genes and (left to right): the pre-treatment FMISO-PET parameters TBR_{peak} (left) and $HV_{1.6}$ as well as the FMISO-PET parameters TBR_{peak} and $HV_{1.6}$ after week two of treatment and residual hypoxia after week two (right).

tions were observed to residual hypoxia in FMISO-PET after week two of treatment ($p < 0.001$).

The median expression of the hypoxia-associated genes evaluated before treatment was calculated individually for the 15-, 26- and 30-gene signature (without the missing gene *DHX34*). Weak correlations with the FMISO-PET parameters evaluated at baseline and in weeks 1, 2 and 5 of treatment were observed (range: −0.07 to 0.43, Table 2). The highest correlations were observed between the median expression of the 15-gene signature and residual hypoxia after two weeks ($\rho = 0.43$) and between the 30-gene signature and $HV_{1.6}$ after week one ($\rho = 0.41$). Twenty-five of the 27 evaluated correlations were positive ($p < 0.001$). Correlations were higher for FMISO-PET after week one and two of treatment compared to baseline and week 5 and for the hypoxic volume $HV_{1.6}$ compared to TBR_{peak} ($p < 0.001$). Supplementary Fig. S1 (top row) shows scatter plots of the median expression of the 15-gene signature with TBR_{peak} and $HV_{1.6}$ at baseline and after week two of treatment as well as with residual hypoxia in FMISO-PET after week 2.

The binary hypoxia classification into less and more hypoxic tumours differed between FMISO-PET and the gene signatures (Supplementary Table S2). Weak correlations were observed (range: −0.12 to 0.38) with the highest correlations between residual hypoxia in FMISO-PET after week 2 and the 15- and 30-gene classifier ($\rho = 0.38$). The classifications of the three hypoxia-associated gene signatures were moderately correlated with each other (range: 0.46–0.70, Supplementary Table S3) and with the corresponding median gene expressions of the signatures (Supplementary Fig. S2).

The median expressions of the hypoxia-associated gene signatures were not correlated to the GTV (Table 2). Moderate correla-

Table 2
Spearman's correlation coefficients between the FMISO-PET parameters TBR_{peak}, $HV_{1.6}$ and residual hypoxia after week 2 as well as tumour volume (GTV) with the median gene expression of the hypoxia-associated 15-, 26- and 30-gene signatures. Correlations significantly different from 0 are marked by *.

Median expression of hypoxia-associated gene signatures		FMISO-PET								
		Pre-treatment		Week 1		Week 2			Week 5	
	GTV	TBR_{peak}	$HV_{1.6}$	TBR_{peak}	$HV_{1.6}$	TBR_{peak}	$HV_{1.6}$	Residual hypoxia	TBR_{peak}	$HV_{1.6}$
15-gene signature	0.26	0.19	0.20	0.35*	0.38*	0.34*	0.36*	0.43*	0.17	0.19
26-gene signature	0.20	−0.07	0.02	0.12	0.22	0.13	0.20	0.27	−0.07	0.06
30-gene signature	0.19	0.14	0.21	0.32	0.41*	0.31	0.33*	0.35*	0.14	0.15

Abbreviations: $HV_{1.6}$, hypoxic volume determined using the standardised uptake value (SUV) threshold 1.6, relative to background; TBR_{peak}, peak tumour-to-background-ratio.

tions (ρ = 0.43–0.69) were found between all FMISO-PET parameters and the GTV (Table 3). These correlations slightly increased up to ρ = 0.72 when in addition to the GTV the median expression of the hypoxia-associated signature genes as well as their interaction with the GTV was included in a generalised linear regression model [Table 3 and Supplementary Fig. S1 (bottom)]. Higher correlations were observed for $HV_{1.6}$ than for TBR_{peak} and correlations decreased for the later time points.

The prognostic value of hypoxia classifiers based on the FMISO-PET parameters and the hypoxia-associated gene signatures regarding LRC and OS was assessed by Cox regression (Table 4). Significantly higher LRC and OS were observed for tumours with smaller TBR_{peak} and $HV_{1.6}$ values at baseline and in week 2 of treatment as well as for tumours with low residual hypoxia, independent of the GTV, as reported previously [19,23]. The hypoxia-associated gene signatures showed a smaller effect size. While less hypoxic tumours had higher LRC using the 15- and 30-gene signature, these differences were not statistically significant. For OS, a significant patient stratification was observed for the 30-gene signature (p = 0.004) and a statistical trend for the 15-gene signature (p = 0.056). The combination of FMISO-PET imaging parameters and the hypoxia-associated gene classifiers was not able to significantly improve the prognosis of LRC compared to FMISO-PET imaging alone using multivariable Cox regression (p > 0.05 in chi-squared tests).

Discussion

Our exploratory study on 42 patients with locally advanced HNSCC investigated the correlations between imaging parameters of four FMISO-PET scans acquired before and during radiochemotherapy and the expressions of three hypoxia-associated gene signatures determined from a local tumour biopsy obtained before treatment. Overall, low correlations were observed which were smaller than the correlations between the FMISO-PET parameters and tumour volume. Concerning LRC and OS, FMISO-PET led to significant patient stratifications at several time-points, independent of the GTV. Classifiers based on the hypoxia-associated gene signatures led to smaller differences in LRC and OS between the patient groups and were not able to improve the prognosis of FMISO-PET imaging.

Gene expressions were determined using a tumour biopsy which contains only a small part of the tumour. Due to intra-tumour heterogeneity, oxygenation of the tumour may differ throughout its volume, and moreover, acute and chronic hypoxia may vary in time, which has been shown in several studies, e.g. using pimonidazole staining [39–42]. Thus, the hypoxia-associated gene expressions may be affected by strong spatial and temporal variations that potentially hide the overall oxygenation status of the tumour. Conversely, FMISO-PET depicts the hypoxic status of the entire tumour volume and can be acquired repeatedly, albeit at a limited resolution (5–8 mm). For FMISO-PET imaging, the parameter TBR_{peak} describes the highest FMISO uptake in the tumour, which is measured in a relatively small volume. It is very unlikely that the biopsy was taken at this particular position. Thus, the correlations may be smaller compared to the parameter $HV_{1.6}$, which describes a larger volume containing voxels with an FMISO uptake above a given threshold. In the present study, in 12 out of 12 comparisons the correlation of $HV_{1.6}$ to the median hypoxia-associated gene expression was larger than that of TBR_{peak} (Table 2, p < 0.001). Still, $HV_{1.6}$ does not necessarily resemble the local oxygenation of the tumour that is evaluated by a biopsy. Besides, for a large cohort of HNSCC xenograft tumour lines imaged with FMISO autoradiography and pimonidazole immunohistochemistry, it was demonstrated that not all microscopic hypoxic areas were visualised by FMISO-PET [43,44], i.e. the biopsy may contain additional information.

The correlation between hypoxia-related molecular expressions or staining intensities and hypoxia PET was considered in a few retrospective studies before. However, they were typically based

Table 3
Spearman's correlation coefficients between FMISO-PET parameters and their prediction by generalised linear models containing the median gene expression of the hypoxia-associated 15-, 26- or 30-gene signature, the logarithm of the gross tumour volume (GTV) and their interaction. For comparison, the correlations between the FMISO-PET parameters and tumour volume alone are shown. All correlations differ significantly from 0.

Median expression of hypoxia-associated gene signatures and tumour volume	FMISO-PET								
	Pre-treatment		Week 1		Week 2			Week 5	
	TBR_{peak}	$HV_{1.6}$	TBR_{peak}	$HV_{1.6}$	TBR_{peak}	$HV_{1.6}$	Residual hypoxia	TBR_{peak}	$HV_{1.6}$
GTV	0.53	0.65	0.59	0.69	0.58	0.58	0.43	0.53	0.44
15-gene signature + GTV	0.60	0.69	0.62	0.71	0.66	0.66	0.49	0.57	0.44
26-gene signature + GTV	0.62	0.67	0.63	0.70	0.61	0.64	0.38	0.64	0.52
30-gene signature + GTV	0.56	0.69	0.60	0.72	0.66	0.67	0.49	0.53	0.44

Abbreviations: $HV_{1.6}$, hypoxic volume determined using the standardised uptake value (SUV) threshold 1.6, relative to background; TBR_{peak}, peak tumour-to-background-ratio.

Table 4
Univariable Cox regression of loco-regional control and overall survival using hypoxia classifiers based on parameters from FMISO-PET imaging and hypoxia-associated genes.

Variable	Loco-regional control		Overall survival	
	HR (95% CI)	p-value	HR (95% CI)	p-value
TBR_{peak} pre-treatment > 2.15	5.31 (1.81–15.6)	**0.002**	2.50 (1.18–5.29)	**0.017**
$HV_{1.6}$ pre-treatment > 19.2 cm^3	5.31 (1.81–15.6)	**0.002**	2.62 (1.24–5.50)	**0.011**
TBR_{peak} week 2 > 2	10.6 (3.09–36.1)	**<0.001**	4.28 (1.87–9.81)	**0.001**
$HV_{1.6}$ week 2 > 4.74 cm^3	6.53 (1.96–21.7)	**0.002**	4.14 (1.86–9.23)	**0.001**
Residual hypoxia $HV_{1.6,week\ 2}/HV_{1.6,baseline}$ >20%	27.2 (3.41–217)	**0.002**	6.02 (2.42–15.0)	**<0.001**
15-gene signature, more vs less hypoxic*	1.57 (0.54–4.52)	0.41	2.08 (0.98–4.42)	0.056
26-gene signature, more vs less hypoxic*	0.65 (0.23–1.88)	0.43	1.08 (0.51–2.29)	0.85
30-gene signature, more vs less hypoxic*	2.22 (0.78–6.34)	0.14	3.11 (1.45–6.71)	**0.004**

Abbreviations: $HV_{1.6}$, hypoxic volume determined using the standardised uptake value (SUV) threshold 1.6, relative to background; TBR_{peak}, peak tumour-to-background-ratio; HR, hazard ratio; 95% CI, 95 percent confidence interval;
* Tumour classification obtained by *k*-mean clustering.

on small patient cohorts without information on the (radio-oncological) outcome [45–53]. In most of these studies, correlations were low as reported here. Sato et al. [48] observed a significant difference in FMISO-PET SUV_{max} between 12 hypoxia-inducible factor 1α (HIF-1α) negative and 11 HIF-1α positive cases of oral squamous cell carcinoma. Weak correlations between FMISO hypoxic volume and HIF-1α ($r = 0.4$) and p53 ($r = 0.47$) were observed by Takashi et al. [47] for 28 head and neck tumours. Spence et al. [46] found no correlations between the vascular endothelial growth factor (VEGF), HIF-1α, Ki67 and p53 evaluated by immunocytochemistry and FMISO HV and TBR_{max} for 22 patients with glioblastoma multiforme, while Kawai et al. [45] observed no or low correlations between HIF-1α, VEGF and FMISO SUV_{max} for 32 patients with glioma. Similar results were observed by Rajendran et al. [54], correlating VEGF expression to FMISO uptake. Also other PET tracers were considered. Mortensen et al. reported no correlations between the 15-gene hypoxia classifier [31] and FAZA PET/CT imaging [7]. Suh et al. [55,56] showed a significant association between the HV in [64Cu](II)-diacetyl-bis(N(4)-methylthiosemicarbazone)-PET and hypoxia gene signatures while no correlation was found for SUV_{max} and TBR for 15 patients with oropharyngeal cancer [50]. A high correlation to HIF-1α staining was observed using the same tracer by Tateishi et al. [57] for 22 patients with glioma. In a meta-analysis comparing the tracers FAZA and FMISO for patients with locally advanced HNSCC treated at four institutions, a high reproducibility of quantitative hypoxia-PET parameters was demonstrated (unpublished).

Correlations between hypoxia-associated gene expressions and FMISO-PET parameters were larger for the FMISO-PET parameters after weeks one and two of treatment than at baseline. This finding is somewhat counterintuitive, since the biopsy was taken before treatment and should reflect hypoxia at that time point, i.e. correlations may be expected largest on the pre-treatment FMISO-PET scan. However, the evaluated hypoxia-associated gene signatures were not developed based on FMISO-imaging and may not only reflect tumour hypoxia but also other radiobiological mechanisms. The signature developed by Toustrup et al. consists of genes that were induced under hypoxic conditions in experimental studies. It has later been used for patient stratification to apply of hypoxic cell sensitizers [31,58]. The hypoxia gene signature by Eustace et al. [32] is based on genes which were initially derived from gene expression analysis compromising 99 genes whose *in vivo* expression was shown to be correlated with 10 well-known hypoxia regulated genes [59]. The third signature was developed by Lendahl et al. and is based on an *in silico* meta-analysis on data sets of the NCBI Gene Expression Omnibus public microarray repository [33]. In the FMISO-PET study, residual hypoxia, defined as the ratio of the FMISO-PET hypoxic volume after two weeks of treatment and pre-treatment, was found to be a strong prognosticator [23]. Residual hypoxia also showed the highest correlation with the expressions of the hypoxia-associated gene signatures and may thus be partially encoded in these signatures. This may be caused by the relation of the hypoxia-associated genes to other biological mechanisms related to radiotherapy response, e.g. stemness and invasive growth [35,60–62].

Recently, the hypoxia-associated gene signatures were evaluated for the retrospective primary DKTK-ROG HNSCC cohort [35]. A statistical trend was observed for lower LRC in tumours with a hypoxic gene expression profile. However, the prognostic power of the signatures was lower than reported previously; e.g. in the DAHANCA 5 cohort the 15-gene hypoxia classifier showed a stronger association with LRC [58]. These differences may arise due to the more advanced disease stage in the DKTK-ROG cohort [35] and differences in tumour localisation between the two cohorts. Also the used method to assess the gene expressions (nanoString) differed from those applied in the original publications defining the signatures and the gene *DHX34* was missing, which may reduce their prognostic value. In the present study, patients with similar characteristics compared to [35] were included, and similar differences in LRC were observed for patient stratifications based on the hypoxia-associated gene signatures. In contrast, FMISO-PET imaging showed larger, significant differences in LRC between classified patient groups, in particular for imaging during treatment. Repeated biopsies or liquid biopsies may play an essential role to improve the prognostic power of hypoxia-associated genes, e.g. by evaluating the change in expression between different time points.

A further promising approach to find potential surrogates for FMISO-PET may be to study correlations to other imaging modalities like CT or functional magnetic-resonance imaging (fMRI), e.g. blood oxygenation-level dependent fMRI (BOLD fMRI) [63]. Recently, the prognostic value of radiomic signatures for LRC was compared between pre-treatment and week two of treatment using computed tomography (CT) scans of the present cohort [64]. The CT-parameters in the signatures (mainly texture features) were moderately correlated to the FMISO-PET parameters TBR_{peak} and $HV_{1.6}$ at baseline and after week two of treatment, showing correlations up to $\rho = 0.6$, i.e. the correlations of CT-imaging parameters to hypoxia FMISO-PET were higher than those of the hypoxia-associated gene expressions.

The findings presented in this study require independent validation, in particular due to the relatively low sample size and the increased risk of false positive results caused by multiple testing. Potential cohorts include the EORTC 1219 – DAHANCA study which recently closed the recruitment of HPV/p16-negative squamous cell carcinomas of the oropharynx, larynx and hypopharynx for accelerated, fractionated RCTx with or without the hypoxic cell radiosensitiser nimorazole [65]. In the associated translational programme, the PET-tracer FAZA as well as the 15-gene hypoxia-associated signature was used for hypoxia evaluation. Moreover, for the Tübingen FMISO image-guided dose escalation randomized phase-II trial using dose-painting by contours, an interim analysis was recently published [66], and the Dresden F-MISO-R trial (DRKS00006007) is currently recruiting.

In this study, low correlations between hypoxia FMISO-PET imaging and the expressions of hypoxia-associated gene signatures were observed for a prospectively collected cohort of 42 HNSCC patients. Higher correlations were obtained to FMISO-PET imaging parameters after week two of treatment and to residual hypoxia after week two. For the prognosis of LRC, FMISO-PET showed a larger effect size than patient classification by the gene signatures. Promising surrogate markers for FMISO-PET may be obtained from more extensive molecular analyses, including repeated tissue biopsies or liquid biopsies, or from additional medical imaging data.

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Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.radonc.2019.02.020.

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