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# Influence of tumor and microenvironment characteristics on diffusion-weighted imaging in oropharyngeal carcinoma: A pilot study

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ABSTRACT

*Objectives:* Diffusion weighted imaging (DWI) is a frequently performed MRI sequence in cancer patients. While previous studies have shown the clinical value of the apparent diffusion coefficient (ADC) for response prediction and response monitoring, less is known about the biological background of ADC. In the tumor microenvironment, hypoxia and increased proliferation of tumor cells contribute to resistance to (radio-)therapy, while high T-cell influx is related to better prognosis. We investigated the correlation between these three tissue characteristics and ADC in 20 oropharyngeal squamous cell carcinoma patients.

*Materials and methods:* 20 patients with oropharyngeal squamous cell carcinoma (OPSCC) who underwent 1.5 T MRI, including DWI were included in this pilot study. Corresponding formalin-fixed paraffin-embedded tumor tissues were immunohistochemically analyzed for protein expression of hypoxia-inducible factor 1a (HIF-1a), Ki-67 and CD3. Expression of these markers was correlated with ADC.

*Results:* ADC negatively correlated with Ki-67 expression ($p = .024$) in tumor cells. There was a significant negative correlation between ADC and CD3-positive cell count ($p = .009$). No correlation was observed between HIF-1a expression and ADC.

*Conclusion:* This study suggests that ADC reflects characteristics of tumor cells as well as the surrounding microenvironment. Interestingly, high tumor proliferation (a negative prognostic factor) and high T-cell influx (a beneficial prognostic factor) are both associated with a lower ADC. Further studies should be performed to correlate ADC to these histological characteristics in relation to previously known factors that affect ADC, to gain further knowledge on the role of DW-MRI in diagnostics and personalized medicine.

## Introduction

In head and neck squamous cell carcinomas (HNSCC) imaging plays a major role in staging, response evaluation and early detection of recurrent disease. Magnetic resonance imaging (MRI) is a modality which is increasingly used, since it provides excellent soft-tissue contrast. Besides conventional anatomical images, additional functional MRI sequences are applied, such as diffusion weighted MRI (DWI). DWI quantifies the restriction of random motion of water molecules in tissues as the apparent diffusion coefficient (ADC) [1,2]. ADC has shown to be useful in differentiating benign from malignant lesions, early treatment response assessment during (chemo)radiation and is promising in prediction of tumor radiosensitivity [3,4].

However, the exact biophysical and biological background of ADC

*Abbreviations:* HNSCC, head and neck squamous cell carcinomas; OPSCC, oropharyngeal squamous cell carcinoma; DWI, diffusion weighted magnetic resonance imaging; TMA, tissue microarray; CI, confidence interval; ADC, apparent diffusion coefficient

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are not yet fully understood. A recent study showed that ADC is correlated to cellular density and stromal components in tumors. However, it is presumed that multiple tissue characteristics may cause restriction of water molecules [5]. It is hypothesized that perfusion and integrity of cellular membranes also affect ADC but evidence of ADC reflected microanatomical characteristics is sparse [6].

The biological properties of a tumor are not exclusively defined by the neoplastic cells but also by the tumor microenvironment which includes immune cells, endothelial cells and tumor-associated fibroblasts [7]. Neoplastic cells and their microenvironment strongly interact: factors such as tumor hypoxia and subsequent necrosis, or proliferation may contribute to variations in the tumor microenvironment. For example, it has been shown that HPV-associated (HPV-positive) HNSCCs have higher levels of tumor-infiltrating lymphocytes [8]. High lymphocyte count was related to improved survival, independent of HPV-status. Another study showed that HPV-positive tumors have lower ADC-values on DW-MRI, which might reflect differences in microenvironment between HPV-positive and HPV-negative oropharyngeal SCC (OPSCC) [1]. We therefore hypothesized that radiological features of a tumor might not only reflect properties of neoplastic cells but also characteristics within the microenvironment. This may also explain the prognostic value of ADC on survival.

We performed a small, exploratory study, combining data from two previously performed studies, to investigate the correlation between ADC, HPV-status and three characteristics of the tumor and its microenvironment: the presence of T-lymphocytes, tumor hypoxia and tumor proliferation, determined by the CD-3 positive cell count, expression of hypoxia-inducible factor 1alpha (HIF-1a) and expression of the proliferation marker Ki-67, respectively.

## Methods and materials

### *Patient selection*

To perform a pilot study on the correlation between tissue characteristics and DWI, two patient databases from previous studies within our institution were combined and resulted in 20 patients who underwent a pretreatment DWI and had tissue available in tissue microarrays (TMAs) [1,9]. While the correlations between histological and DWI data on clinical outcome have been described separately before, the present study describes the correlations between the histology and imaging data. Pre-treatment MRI, including DWI had been performed in a cohort of 75 consecutive patients with a first primary, histopathologically proven HNSCC, treated in our center with (chemo)radiotherapy with curative intent from April 2009 to August 2011. Inclusion criteria were T2, T3 and T4 cancers located in the oral cavity, oropharynx, hypopharynx or larynx. These MRI-scans (including DWI) were part of routine pretreatment imaging.

Tissue from 20 of the 75 the aforementioned patients was available in TMAs created for previous studies [9,10]. Briefly, these were cohorts of 274 OPSCC patients with a first primary OPSCC between 1997 and 2010 in our center. For all studies, follow-up data were obtained at routine outpatient clinic visits. In both studies, leftover material from routine diagnostics was used and obtaining informed consent was not necessary according to laws and 'Best Practice' guidelines in our country. HPV-status was determined by immunohistochemical staining for p16, followed by a molecular HPV-detection test when positive [11,12].

### *Magnetic resonance imaging protocol*

All MRI scans with DWI sequence had been performed for radiotherapy planning purposes. MRIs were acquired on a 1.5 T MRI scanner with 2 surface coils. (Intera NT, Philips Medical Systems, Best, The Netherlands). The MRI protocol consisted of transverse T1-weighted turbo spin echo before and after injection of gadolinium. Transverse and coronal T1-weighted turbo spin echo after gadolinium with spectral presaturation with inversion recovery (SPIR) fat suppression. Transverse and coronal proton density with a short tau inversion recovery (STIR) fat suppression. Included was a transverse diffusion-weighted MRI. Diffusion weighting was achieved by using a single-shot spin-echo planar imaging sequence (TR/TE: 5872 ms:70 ms; EPI factor 51), with a STIR fat suppression with an inversion time of 180 ms and diffusion weighting in three orthogonal directions with b-values of 0, 150, and 800 s/mm$^2$. Images were acquired with a 112 × 101 matrix, an acceleration factor of 2, a slice thickness of 4 mm, and a slice gap of 0 mm; the number of averages was four. ADC was calculated using all three *b* values. The 3D tumor-volume was manually delineated on the axial slides with a *b* value of 0 s/mm$^2$ by using the additional information of all other MR images by an experienced head and neck radiologist and an ENT resident in consensus, both having over 5 years of experience with DWI. Evidently necrotic or cystic areas were separately delineated and subtracted from the total tumor volume.

### *Immunohistochemical analysis*

TMAs were constructed and immunohistochemical (IHC) staining was performed as previously described [13,14]. Briefly, representative areas of tumor were marked on hematoxylin and eosin (H&E) stained sections of pre-treatment tumor tissue biopsies, by a dedicated head and neck pathologist. Three 0.6 mm tissue cores per patient were then extracted from the original paraffin block and introduced in the recipient TMA block. Four micrometer sections were stained for Ki-67 and CD-3 protein expression using a Ventana Autostainer (Ventana Medical Systems, Inc, Tucson, USA) and for HIF-1a using a manual staining procedure using the Novolink Kit (Leica Biosystems, Eindhoven, the Netherlands) according to methods described previously [9]. Briefly, slides were deparaffinized and rehydrated, followed by blocking of the endogenous peroxidase activity, antigen retrieval, and incubation with the primary antibody as shown in Table S1. After incubation with the secondary antibody (OV HRP Multimer, Ventana Medical Systems, 8 mins for CD3, Ki-67, Novolink Polymer, 30 mins for HIF-1a), the slides were developed using diaminobenzidine (DAB) and counterstained with hematoxylin, followed by dehydration and coverslipping. On every TMA, normal tonsillar tissues were included as controls. In addition, for every manual staining procedure for HIF-1a, renal cell carcinoma tissue was included as a positive control, and as a negative control by incubation with PBS-BSA instead of the primary antibody.

The stained sections were reviewed by a dedicated head and neck pathologist and an otorhinolaryngology resident in consensus, who were unaware of the clinical data. For CD3, the number of CD3-positive stained cells was manually counted at 400x magnification. Because the TMA-cores were similar in size, normalizing the number of cells for the area was not necessary. For HIF-1a and Ki-67, the percentage of positive stained tumor cells was scored at 200x magnification for each core. Only nuclear staining was considered positive for HIF-1a and Ki67. A mean score of the three cores was calculated for each staining and used for further analyses. Cores were excluded if they could not be evaluated because of folding, when they were missing or when there was less than 5% tumor tissue present in a core.

### *Statistical analysis*

Normality of the variables HIF-1a protein expression, Ki-67 protein expression, CD3 positive staining cells and mean tumor ADC was tested using the Shapiro-Wilk test. In none of these variables, the null-hypothesis of being normally distributed was violated. Correlations between histological data and ADC were analyzed using Pearson correlation with bootstrapping (1000 samples) to provide confidence intervals (CIs). For visual representation of the data, we performed univariate linear regression. P-values below 0.05 were considered statistically significant. All statistical analyses were performed in SPSS

**Table 1**
Baseline patient and tumor characteristics.

| | OPSCC |
|---|---|
| Sex | |
| Male | 11 (55) |
| Female | 9 (45) |
| Age | 61.4 (9.3) |
| Clinical T-stage | |
| T2 | 8 (40) |
| T3 | 4 (20) |
| $T4_{a/b}$ | 8 (40) |
| Clinical N-stage | |
| N0 | 3 (15) |
| N1 | 3 (15) |
| $N2_{a/b/c}$ | 14 (70) |
| N3 | – |
| HPV-status | |
| Positive | 4 (20) |
| Negative | 16 (80) |
| CD3 count | 124 (78) |
| % HIF-1a expression | 32 (27) |
| % Ki-67 expression[a] | 32 (19) |
| ADC ($\times 10^{-3}$) | 1.53 (0.31) |
| Total *n* (%) | 20 (100) |

Categorical variables are shown as *n* (%), continuous variables as mean (SD).
[a] Ki-67 expression could not be determined for 1 patient.

version 22.0 (IBM). Graphs were constructed in Graphpad Prism version 6 (Graphpad Software, Inc.).

This manuscript adheres to the STROBE statement, or checklist of items that should be included in reports of observational studies [15]. This checklist is included as Supplementary data.

## Results

Tissue and imaging data were available for 20 patients. Baseline patient characteristics are shown in Table 1. These were 9 women (45%) and 11 men (55%) with an average age of 61.4 years (SD = 9.3). Seventeen patients (85%) had lymph node metastases.

All patients were primarily treated with radiotherapy, six (30.0%) in combination with platinum based chemotherapy, six (30.0%) in combination with cetuximab. Two patients (10%) underwent a neck dissection prior to radiotherapy alone. HPV-status was positive in 4 tumors (20%). Because of the low number of HPV-positive patients, no separate statistical analyses were performed in this subgroup.

HIF-1a and CD3 staining was available for all patients, Ki-67 staining was available for 19 of the 20 patients (95%). The raw scores per TMA core and ADC-value are shown in Supplementary Table S2. The number of CD3-positive cells per 0.6 mm core was on average 124 (range 17–267, SD = 78). Mean Ki-67 and HIF-1a positive cells were respectively 32% (22.5–85%, SD = 19%) and 32% (1–80%, SD = 27%). Examples of the staining patterns are shown in Fig. 1. The mean whole tumor ADC was $1.53 \times 10^{-3}\,mm^2/s$ (range $1.18–2.28 \times 10^{-3}\,mm^2/s$, SD = $0.31 \times 10^{-3}$). An example of a DWI-scan is shown in Fig. 2.

The linear regression analyses between histological characteristics and the ADC values is shown in Fig. 3. The following correlation coefficients were obtained using Pearson correlation after bootstrapping. There was a strong, inverse correlation between Ki-67 expression and the mean tumor ADC (r = −0.514, 95% CI −0.795 to

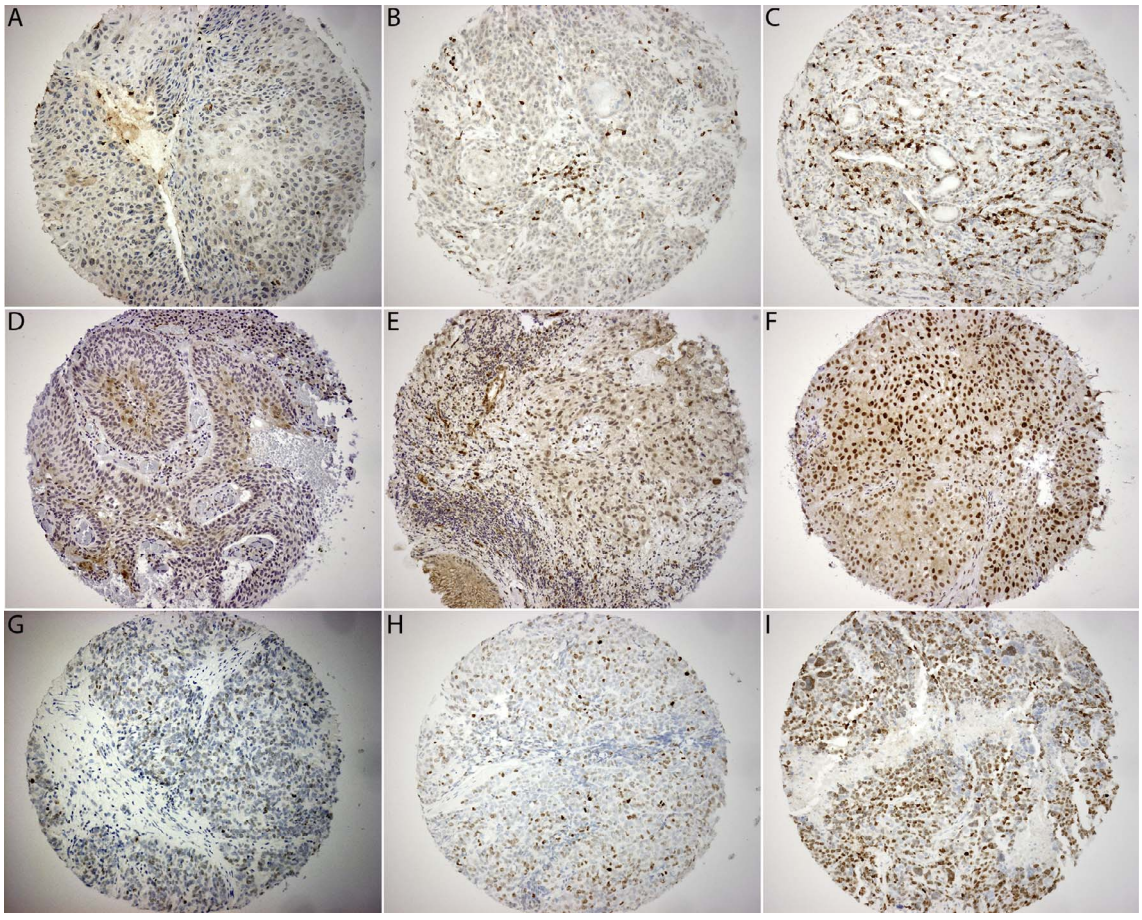


**Fig. 1.** Immunohistochemical staining, Staining examples of CD3 (A-C), HIF-1a (D-F) and Ki-67 staining (G-I). In A, B and C, there are 5, 70 and 280 CD3 + positive cells, respectively. D shows 5% HIF-1a positive tumor cells, E and F show 20 and 80% of HIF-1a positive tumor cells. G, H and I show 10, 25 and 80% Ki-67 expressing tumor cells.

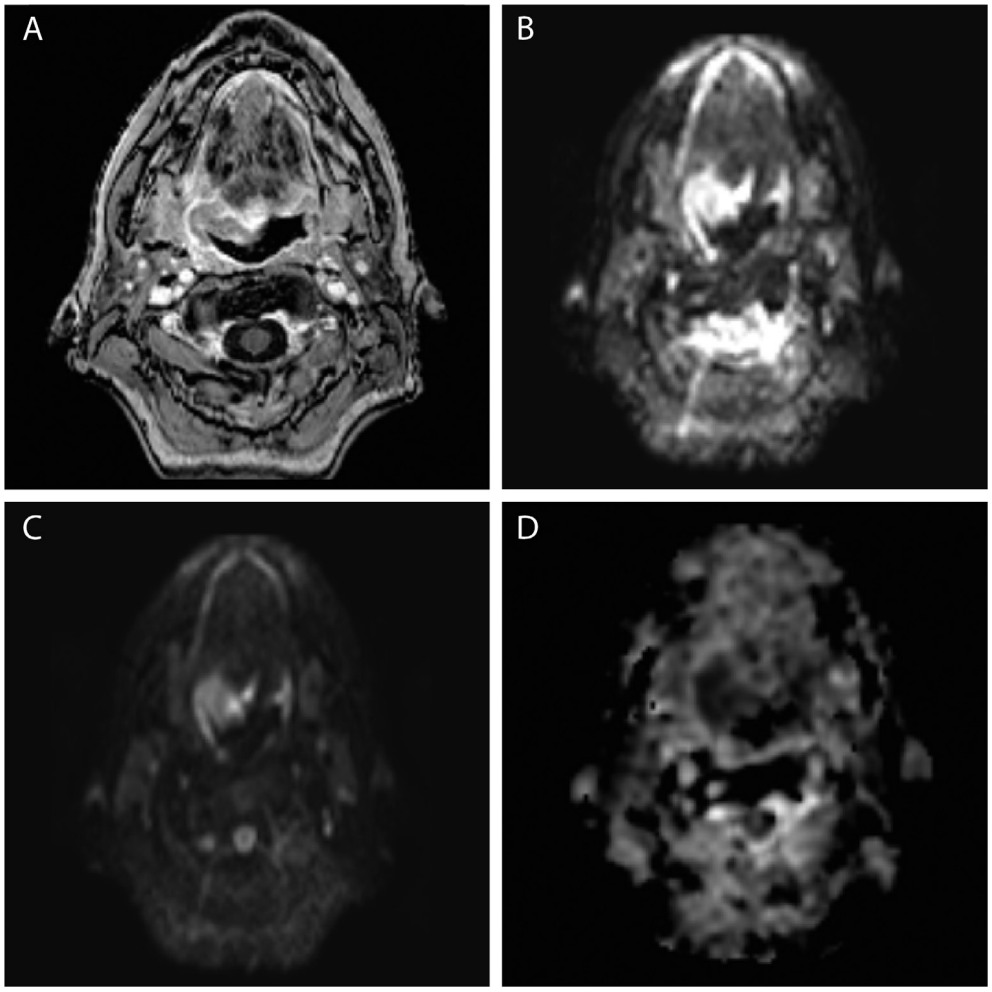


**Fig. 2.** Diffusion-weighted imaging, Axial MR images in a 61 year-old male with an oropharyngeal carcinoma centered in the right base tongue. Axial post-contrast T1-weighted MR image with fat suppression (a), axial DW image $b = 0\,s/mm^2$ (b) and $b = 800\,s/mm^2$ (c). The corresponding axial ADC map is shown in (d).

−0.033, p = .024). There was a significant correlation between the ADC and CD3 positive cell count (r = −0.568, 95% CI −0.809 to −0.263, p = .009). There was no significant correlation between HIF-1a expression and the ADC (r = 0.356, 95% CI −0.079 to 0.718, p = .123). As for some patients only a single tissue core was available (see Table S1), sensitivity analyses were performed including only patients with more than one tissue core available. Including only patients with three available cores yielded similar results compared to the analyses with all patients (*data not shown)*.

## Discussion

The goal of this small, exploratory study was to investigate the biological background of ADC values obtained with diffusion-weighted imaging, by correlating ADC with characteristics of the tumor and tumor microenvironment for OPSCC. We investigated three factors: T-lymphocyte influx, tumor hypoxia and tumor proliferation. We found that tumor proliferation had a strong and significant inverse correlation with tumor ADC and that T-cell count inversely correlated significantly with ADC. We believe these characteristics should be further investigated, along with previously known tissue characteristics that influence ADC.

DWI reflects water mobility on a microscopic level. Although numerous studies have demonstrated the use of DWI in the prediction of tumor radiosensitivity and early treatment response assessment, [3,4] little is known about the biophysical background of DWI and an explanation to why ADC is able to predict outcome remains unclear. There are some hypotheses for the predictive potential of ADC for treatment outcome; ADC is reported to correlate with cellularity, stromal component, nucleus-to-cytoplasm ratio and HPV status [1,5,16,17]. These factors have all been described to influence patient outcome [18–23]. However, radiosensitivity of a tumor is not only based on tumor cell characteristics, but also based on factors within the tumor microenvironment, such as T-lymphocyte influx, vascularity or hypoxia. The variation in ADC and the correlation with prediction of radiosensitivity and treatment response might be explained by the microenvironment. The present study gives insight in the relation of ADC and three tumor characteristics factors which are proven to relate to outcome [8,24–26]. These correlations will help elucidate the complex reflection of ADC of the tissue on a biological and biophysical level.

Ki-67 expression is a proliferation marker which is expressed during all phases of the cell cycle, with exception of G0. Previous studies have described that high expression of Ki-67 is associated with worse survival and with higher chances of lymph node metastasis compared to tumors with lower Ki-67 expression [25–28]. In the present study, we observed a significant inverse correlation between ADC and the percentage of Ki-67 expressing cells. This has also been observed in a small number of studies of tumors from different histologies, including CNS, rectal or breast malignancies [29–32]. To our knowledge, the present study is the first to report similar results in HNSCC, or in squamous cell carcinoma in general.

The finding that high Ki-67 expressing tumors have lower ADC may be explained by biomechanical reasons: High cell proliferation may lead to a higher cell density and, as a result, less stroma, both of which may cause more diffusion-restriction of water molecules, leading to lower ADC. Also, because ADC is highly influenced by diffusion of

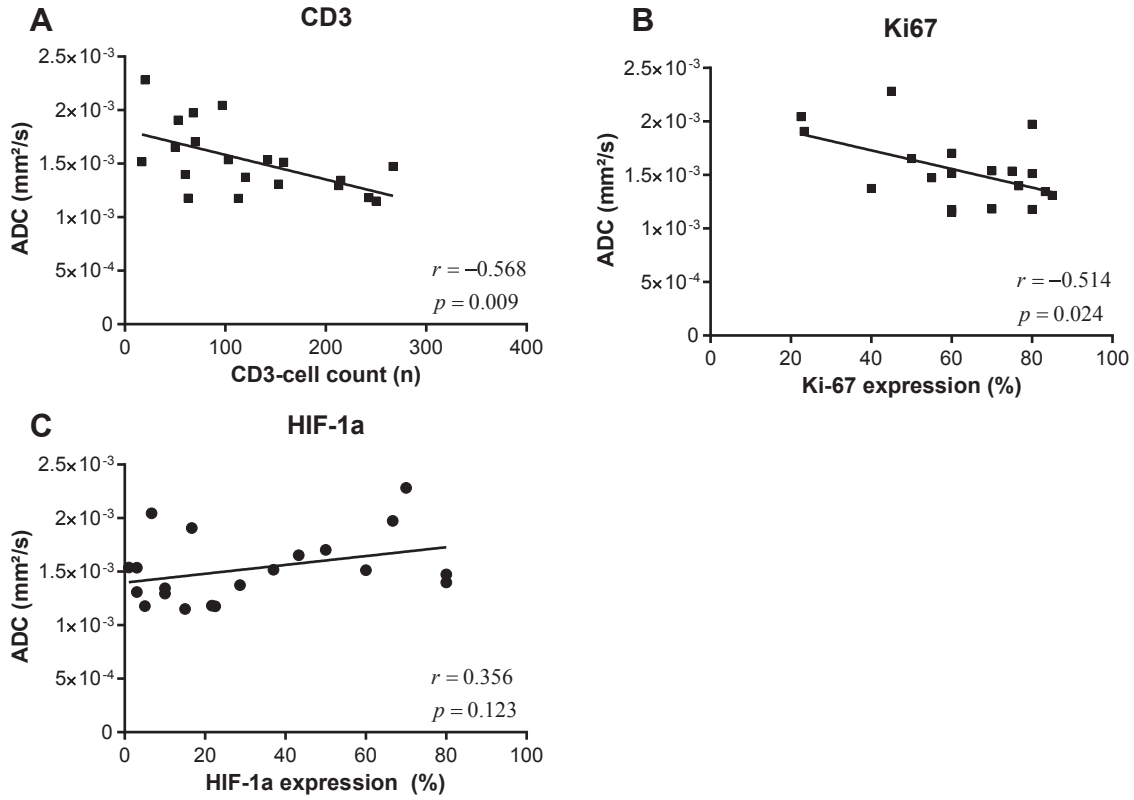


**Fig. 3.** Correlations between histological markers and DW-MRI, A significant inverse correlation was observed between CD3-cell count and ADC (A). Ki67 significantly inversely correlated to ADC (B). No correlation was observed between HIF-1a expression and ADC (C).

water molecules within the tumor stroma [5], larger cell-to-stroma ratios due to proliferation may lead to lower ADC.

Another parameter we investigated was T-lymphocyte influx. We hypothesized that lymphocyte influx is associated with decreased ADC. Lymphocytes are small and have a high nucleus-to-cytoplasm ratio. Therefore, high numbers of tumor-infiltrating lymphocytes should theoretically lead to lower ADC values. Indeed, we found that higher lymphocyte counts were associated to lower ADC in OPSCC. While the oropharynx is rich in lymphocytes in general, there may be biophysical differences between subsites causing different relations between ADC and lymphocyte infiltration. Therefore the present finding should be further studied across other subsites.

Both higher Ki-67 expression and higher T-lymphocyte influx were associated with lower ADC. This is interesting as tumor proliferation correlates to poor prognosis, while the presence of immune cell infiltrates is associated with a better prognosis. Most studies in HNSCC patients describe a favorable prognosis for tumors with low ADC [3,4,33,34]. One could argue that in the oropharynx, the beneficial effects of high tumor immune cell infiltrate weighs against the effects of proliferation on patient survival. In fact, only a single study suggests that Ki-67 expression has prognostic value in OPSCC [35], while other studies did not find such an effect in this subsite [36–38]. Exactly this contradictory finding highlights the importance of understanding how the ADC is established on a microanatomic level. Such an hypothesis should be investigated with multivariate analysis of the effect of proliferation and immune cell invasion on both the ADC and on prognosis.

In addition, it is often hypothesized that high ADC values in tumors might reflect microscopically necrotic or hypoxic areas [39]. During chronic tumor hypoxia, cellular survival mechanisms are activated within the tumor, leading to lower treatment sensitivity and decreased survival for patients with head and neck cancer [24]. In the present study we observed no association between expression of the hypoxia related protein HIF-1a and ADC. This suggests that hypoxia alone will not always lead to necrosis, apoptosis or other cellular states that will decrease restriction of the diffusion of water molecules sufficiently to affect the tumor ADC. This is supported by a previous study, where no differences in necrosis were found between tumors with high ADC versus tumors with low ADC [5].

Alternatively, because of tumor heterogeneity it may be argued that only certain regions within the tumor are hypoxic [40]. Therefore, the use of biopsies, as well as a mean ADC value for the whole tumor may not be reliable enough to investigate an actual relation between DWI and hypoxia. In addition, the presence of tumor hypoxia could be investigated using a hypoxia gene expression profile, such as described by Toustrup and later confirmed by Tawk [41,42]. Also, the effect of hypoxia on ADC could have been too small to detect in this small pilot study. Immunohistochemical analysis of whole tumor slides in a larger cohort, to perform spatial correlations between biomarkers and imaging may possibly be more appropriate to investigate this relation.

Several points should be addressed. In this exploratory study, we combined data from two previous studies to investigate the underlying biology of the ADC. While we consider it a strength to combine data from various field of research, this is also a limitation. The final sample size was small, because of different in- and exclusion criteria in each study. However, the study did provide several findings that deserve further and multivariate investigation in larger patient cohorts.

Due to the small sample size, it was not possible to compare ADC values between HPV-positive and HPV-negative patients in relation to the other tissue characteristics. Larger studies should also further clarify the relation between HPV-status and DWI, as the studies on this subject vary in outcome [1,43,44]. Two studies describe a significant difference in ADC between HPV-positive and HPV-negative patients [1,44], while another study does not [43]. There are histological differences between HPV-positive and HPV-negative tumors, which could suggest that there are also differences within the microenvironment. It would be interesting to see whether differences in ADC-values between HPV-positive

and HPV-negative OPSCC tumors retain their significance when corrected for factors such as proliferation or lymphocyte-influx.

In future studies, it would be interesting to perform voxel-by-voxel, or spatial correlations between tissue characteristics and imaging. Because we included radiotherapy-treated patients and used only tissue biopsies to assess immunohistochemical characteristics, this was not possible in the present study. Such correlations might be investigated in studies where patients undergo imaging, as well as surgical resection of the tumor, for instance using a design as was used in a previous study [45].

To summarize, we have found that ADC is influenced by tumor characteristics (*proliferation*), but also factors within the tumor microenvironment (*immune cell influx*). Interestingly both these characteristics have a similar correlation with ADC, even though immune cell influx is considered a beneficial prognosticator, while proliferation is not. Better understanding of the microanatomical basis of ADC will provide clinicians with better understanding of biological and biophysical properties of tumors at a cellular level. Ultimately, this study contributes to discovering the mechanism and role of DWI and ADC values for diagnostic and prognostic purposes in HNSCC.

## Conflict of interest

No conflict of interest is declared by all authors.

## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.oraloncology.2017.12.001.

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