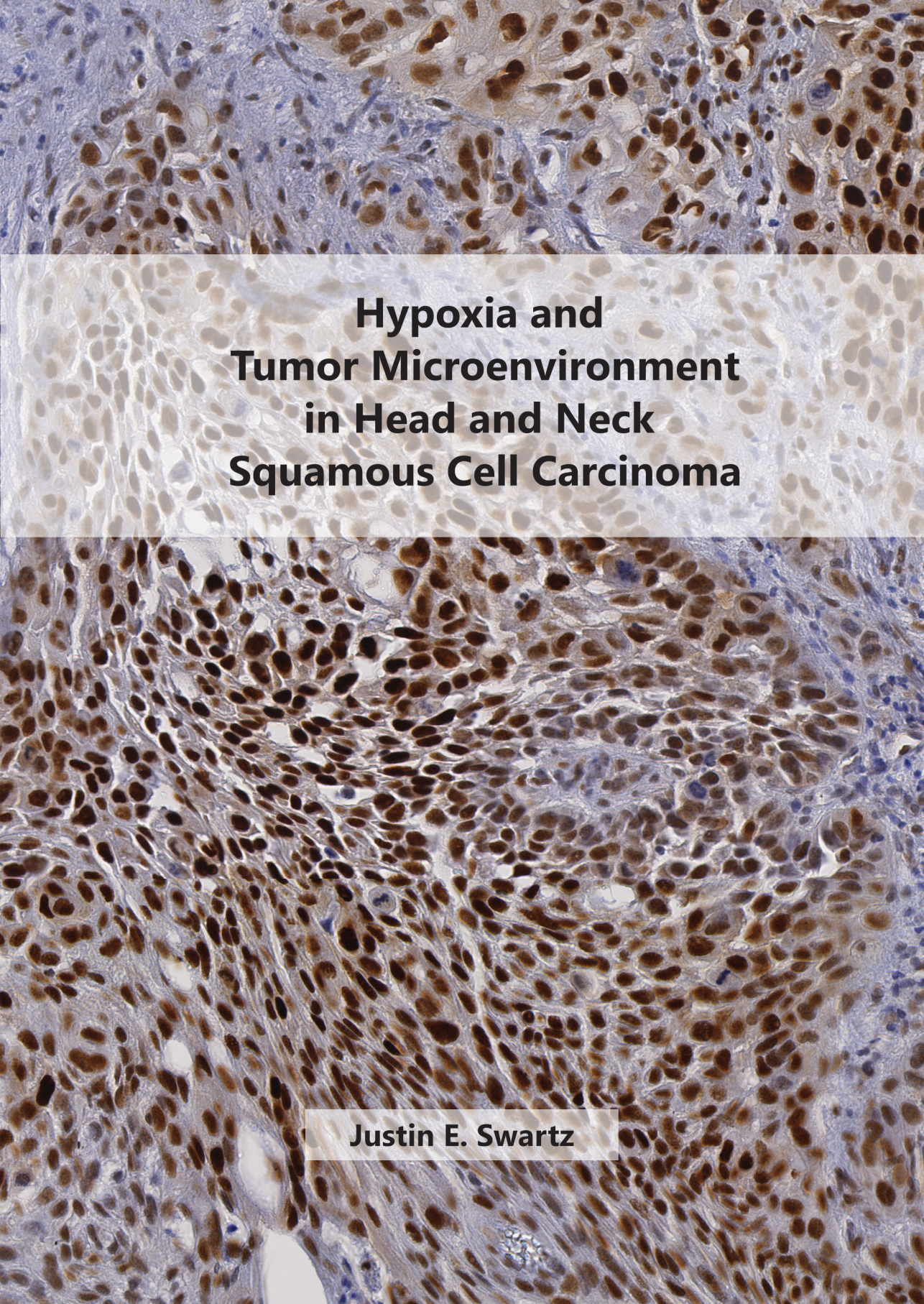

Hypoxia and Tumor Microenvironment in Head and Neck Squamous Cell Carcinoma

Justin E. Swartz

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Hypoxia and tumor microenvironment in head and neck squamous cell carcinoma

Hypoxie en tumor micro-omgeving in
plaveiselcelcarcinomen in het hoofd-halsgebied
(met een samenvatting in het Nederlands)

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Chapter 1

General introduction

Head and neck cancer

Head and neck squamous cell carcinoma (HNSCC) comprises carcinomas arising from the epithelium of the upper aerodigestive tract (**Figure 1**). The most important risk factors for developing HNSCC are smoking and alcohol use.[1,2] Recently, the human papillomavirus (HPV) has been established as another, separate risk factor for oropharyngeal squamous cell carcinoma. As the head and neck region is important both functionally and esthetically, the potential effects for the patients by both the tumor and the treatment are considerable.

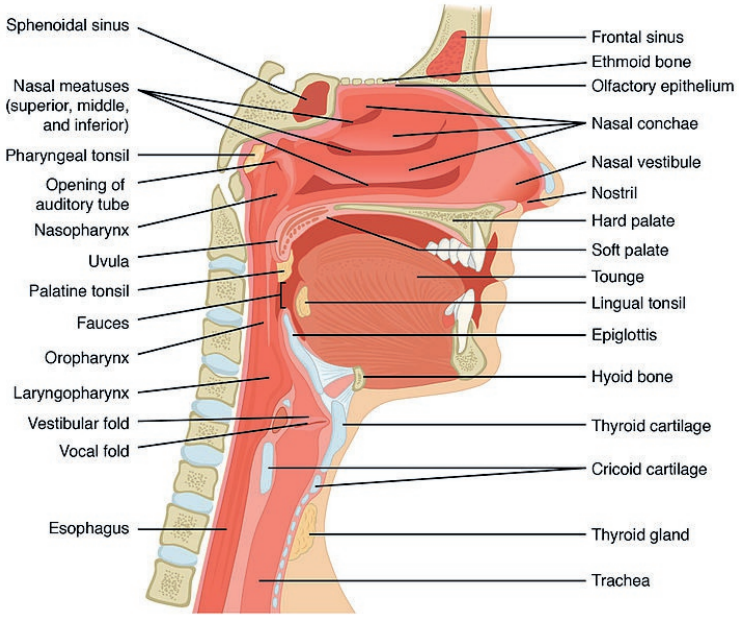

Figure 1 | The anatomy of the head and neck region. *Image reused from the book "Anatomy and Physiology" by OpenStax.*[3,4]

Anatomy

Several sites are distinguished within the head and neck region. The oral cavity includes the buccal mucosa, mobile tongue, upper and lower gingiva, the hard palate and the floor of the mouth. The dorsal third of the tongue is considered the base of the tongue and belongs to the oropharynx, along with the vallecula, tonsils, tonsillar fossa and pillars, inferior surface of the soft palate, uvula and posterior from the level of the junction between hard and soft palate cranially until the level of the hyoid bone (or tip of the epiglottis) caudally.

Below the oropharynx is the hypopharynx, that includes the piriform sinuses, post-cricoid region and its posterior wall. The voice box, or larynx, separates the airway from the 'food pipe' or esophagus and is divided in a supraglottic, glottic and subglottic region. The supraglottis consists of the ventricular folds, the laryngeal ventricle, the arytenoids, epiglottis and aryepiglottic folds. The glottis consists of the vocal cords and the subglottis is the area caudally from 1 cm below the vocal cords. Above the oropharynx is the nasopharynx, and the region anterior to the nasopharynx (separated by the choana) is considered the nasal cavity.

Epidemiology

With an incidence of around 3000 new cases in the Netherlands, HNSCC is on the 8th place of most common cancers in men and 9th place in women and the incidence has risen in the last years (**Figure 2**).[5] HNSCC is mainly located in the oral cavity (OSCC, 36.5%), followed by larynx (LSCC, 29.4%) and oropharynx (OPSCC, 10.7%).[6]

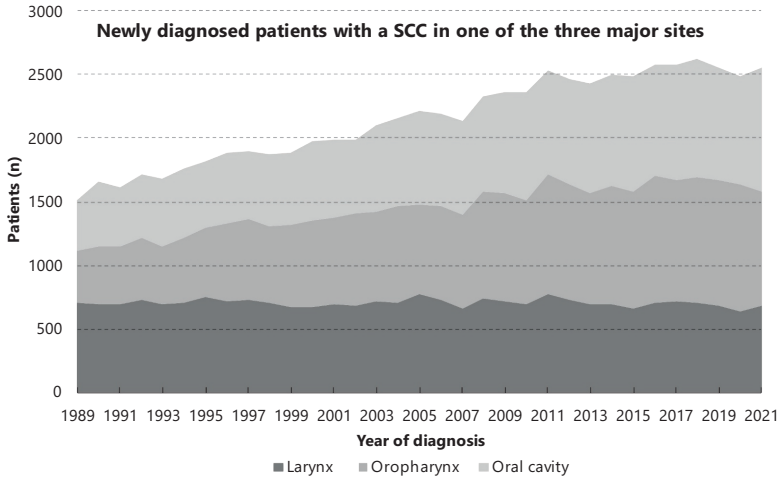

Figure 2 | Incidence of OSCC, LSCC and OPSCC in the Netherlands 1989 – 2021. Data from 2020 and 2021 are still preliminary. *Figure based upon data from the Dutch Cancer Registry.*[5]

The stage at the time of diagnosis can be divided into early, or local disease (stage I-II) or advanced disease, which includes locally advanced disease and locoregional disease (stage III- IVB). OSCC and LSCC are often diagnosed in stages I/II, but OPSCC and hypopharyngeal squamous cell carcinoma (HPSCC) are mostly diagnosed in stages III or IV. In the case of distant metastasis the stage is IVC.

The prognosis of patients with HNSCC is relatively poor, and differs per site and disease stage at diagnosis. In recent years, advances in therapy have improved the prognosis and survival of patients only minimally.[6] In the Netherlands, 5-year survival rates of the three large subcategories have increased from 57% in the period 1991-2000 to 62% in the period 2011-2019 for patients with OSCC. For OPSCC the increase is from 36% to 52%. The survival of LSCC has remained stable around 68%.

Current treatment paradigm

Regular treatment options for HNSCC generally include surgery, and organ-preservation treatment in the form of radiotherapy. In some cases of advanced disease, platinum based chemotherapy is added as a radiosensitizer.[7] When the patient is not fit for chemotherapy and in HPV-negative disease the monoclonal antibody against the epidermal growth factor receptor (EGFR) cetuximab may be used instead.[8]

Generally, performing single-modality treatment with surgery or radiotherapy is pursued in stages I and II and selected cases of stage III disease. However, in most advanced cases, multimodality treatment is required. This could be surgery followed by adjuvant (chemo)radiotherapy or primary radiotherapy with platinum-based chemotherapy and salvage surgery in reserve for residual disease. The choice of therapy is based on the disease extent, the disease site and the expected loss of function by the therapy. Several landmark trials, such as the Vetaran Affairs Laryngeal Cancer Study Group trial, have established chemoradiation as an alternative with a higher chance of organ preservation and comparable survival in some, but not all cases.[9–11]

Novel treatment paradigms

Would it not be more logical that the choice of the treatment of cancer patients is not based on the location of the tumor but on its biological properties? In the 1980's researchers have already tried to culture human tumors in a comparable way to bacteria.[12] The goal was to establish *in vitro* which chemotherapeutic drug would be most successful at eradicating the tumor. Unfortunately, this approach has not found its way into the clinic yet in this exact form as it was hard to culture tumor tissue *in vitro*.[13] Moreover, the correspondence between *in vitro* and *in vivo* treatment outcomes was not sufficient. Promising results have been reported by testing chemotherapeutic drugs and irradiation on organoids derived from HNSCC patients, although this technique is not yet ready to predict treatment outcome in clinical practice.[14]

One important discovery toward tumor biology-based treatment choice concerns the HPV-related (HPV+) OPSCC. This subgroup of OPSCC is a distinct subgroup of tumors with a favorable prognosis compared to smoking and drinking-related OPSCC.[15] In trials and retrospective cohorts, patients with HPV+ OPSCC have a much better prognosis compared

to patients with non HPV-related (HPV-) tumors.[16] Therefore trials are currently ongoing to see whether treatment de-intensification can be safely performed in patients with HPV+ tumors.[17] The distinction in HPV+ from HPV- OPSCC has been recently introduced in the 8th TNM classification as well. As an example, since TNM8 some tumors that may be considered T1N3b (stage IVB) when HPV- are considered T1N1 (stage I) when HPV+, illustrating a beneficial prognosis for HPV+ tumors.[18]

Another important change concerned the view on tumor biology. In another landmark paper, Hanahan and Weinberg proposed that tumors should not be viewed as a lump of homogenic tumor cells (*"The Reductionist View")*, but as a heterogeneous micro-environment of tumor cells, cancer stem cells, immune cells, blood vessels, stroma and other cell types (*"A Heterotypic Cell Biology"*, **Figure 3**).[19,20] Some of the factors in the microenvironment interact to improve survival and therapy resistance and were termed hallmarks. These hallmarks may be exploited singular or in combination with novel therapies (**Figure 4**). One emerging therapy that exploits such a hallmark and has already found its way into clinical practice is immunotherapy, which will be further elaborated later on in this chapter. As hypoxia plays a part in several of these hallmarks, such as resisting cell death, avoiding immune destruction and inducing angiogenesis, targeting hypoxia would result in targeting multiple hallmarks at once.

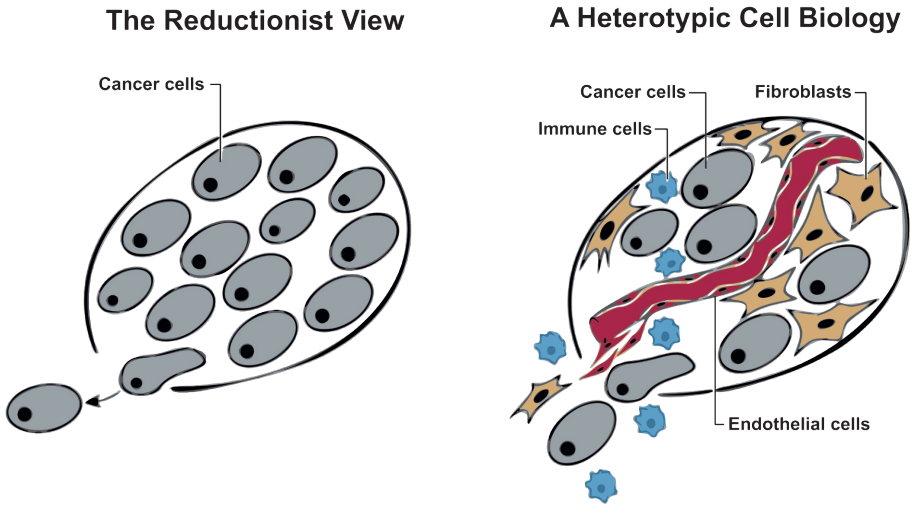

Figure 3 | Comparison of the 'reductionist' view and the 'heterotypic tumor cell biology' as proposed by Hanahan and Weinberg. *Image from Hanahan and Weinberg.*[19]

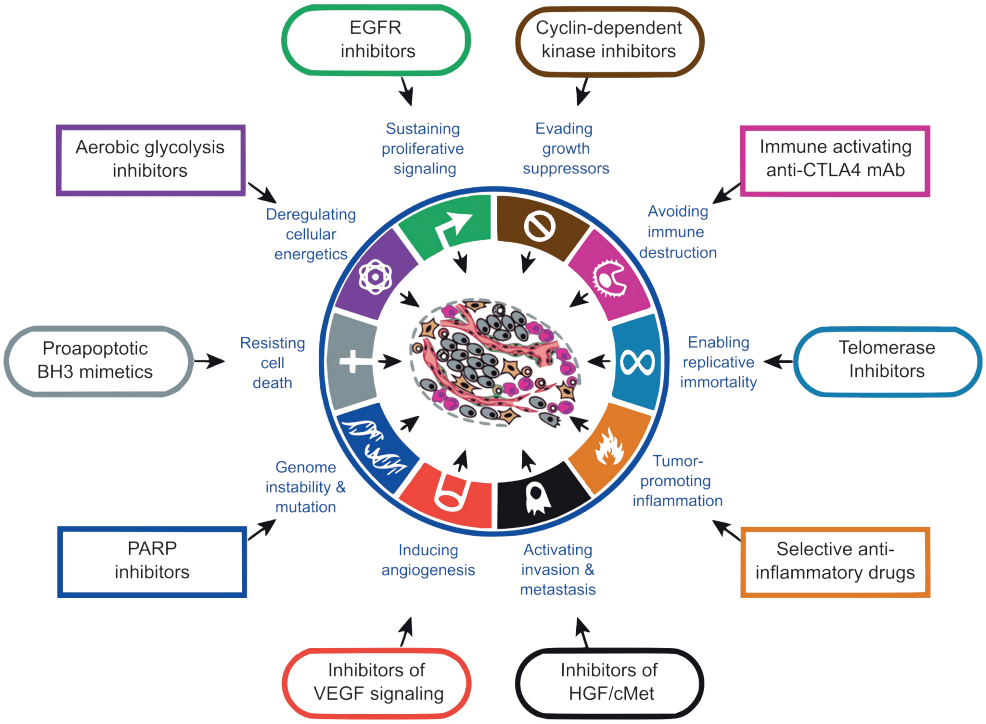

Figure 4 | The "Hallmarks of Cancer" model by Hanahan and Weinberg with illustrative examples of therapies directed at these hallmarks. *Image from Hanahan and Weinberg.* [20]

Tumor hypoxia

Hypoxia is a characteristic of many solid tumors.[21] By definition it is a mismatch between oxygen supply and demand. There is a distinction between acute (or: *perfusion-limited*) and chronic (or: *diffusion-limited*) hypoxia, although both forms may co-exist within tumors.[22]

Perfusion-limited (acute) hypoxia

Acute hypoxia, or perfusion-limited hypoxia, is usually a temporary state that occurs because of compromise of blood supply to the tissue. Oxygen homeostasis is an important part of homeostasis in general. Therefore, mechanisms exist to counteract acute and chronic hypoxia. For acute hypoxia, several mechanisms are present in different parts of the body to cause vasodilation in the case of hypoxic circumstances.[23] Systemic hypoxia may be detected in arterial blood by the glomus cells in the carotid body, leading to systemic vasodilation.

Perfusion-limited hypoxia may occur locally for instance when afferent blood vessels are temporarily compressed (due to external pressure, positioning) or clamped (iatrogenic). In a smaller scale within tissues or within a tumor, perfusion-limited hypoxia is thought to arise primarily from vascular stasis (by vascular collapse, occlusion by tumor cells or leukocytes), flow instabilities or increased interstitial fluid pressures. [24–26]

Diffusion-limited (chronic) hypoxia

Diffusion-limited hypoxia is a state where the distance between cells and the nearest blood vessels is too large for sufficient amounts of oxygen to diffuse to these cells. This may arise because a tumor cell outgrows it vascular supply. Moreover, because tumors often exhibit inefficient, chaotic vascular patterns, the distance of a cell to the nearest blood vessel may also be quite variable. Cells with increased distance to the nearest blood vessel are therefore exposed to increased levels of hypoxia.

Transient hypoxia

While theoretically this distinction between acute and chronic hypoxia holds true, *in vivo*, the distinction is less obvious.[24] In fact perfusion-limited and diffusion-limited hypoxia may coexist within the same tumor because of the suboptimal vascular patterns.[27] Moreover, hypoxia may also be transient and this variant may possibly be most relevant clinically. A simulation study showed that as much as 25% of a tumor region may temporally fluctuate above and below a certain threshold of hypoxia.[28]

Hypoxia and treatment resistance

Hypoxia is a common trait of solid tumors as irregular, exponential growth of tumor cells leads to a tumor outgrowing its own vascular supply causing diffusion-limited hypoxia. As a consequence, the stimulation of angiogenesis by factors such as VEGF lead to the formation of vessels with suboptimal architectures, causing perfusion-limited hypoxia.[27,29] Thus, both these forms of hypoxia may co-exist within a single tumor (**Figure 5**).

Clinically, hypoxia has been known to decrease sensitivity to anti-tumor treatment. The most well-known treatment effects are described in radiotherapy.[22] It has been established in 1935 that benign or malignant hypoxic tissues are less sensitive to radiation than normoxic tissue and that the decrease in radiosensitivity is around three-fold.[30–32] Partly this is attributed to a direct effect of radiation on oxygen: oxygen is required for the formation of free radicals to induce double strand DNA breaks.[21] The same mechanism of reduced generation of free radicals leads to treatment resistance for some forms of chemotherapy (including bleomycin and doxorubicin) and photodynamic therapy.[33] The critical O_2 tension (pO_2) below which radiotherapy resistance occurs is around 25-30 mmHg and pO_2 levels below 0.5 mmHg lead to maximal radiation resistance. As a reference, the mean arterial pO_2 in the human body is 90 +/-5 mmHg, the mean venous pO_2 is 40+/- 5 mmHg and the pO_2 of

brain tissue lies between 30 and 48 mmHg.[34] Interestingly, the pO_2 of normal skin (not mucosa) is already below the critical level for radiotherapy resistance with a mean of 8 mmHg.

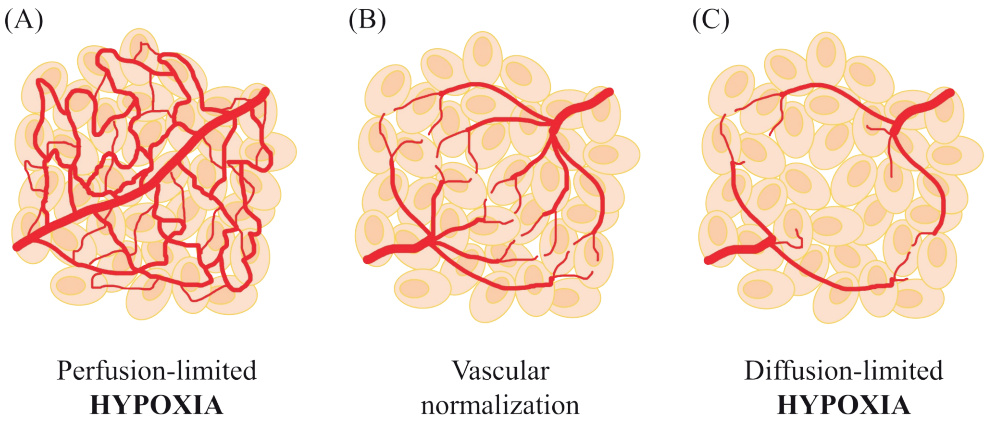

Figure 5 | Perfusion-limited versus diffusion limited hypoxia. Example of perfusion limited hypoxia caused by irregular vessel formation (A), normal vascularization (B) and diffusion limited hypoxia as a result of insufficient vascularity (C). *Image from Codony et al.*[27]

Apart from this direct effect of hypoxia in inducing therapy resistance, there is also an indirect effect by changes in protein expression by hypoxic cells. Hypoxia leads to accumulation of the transcription factor Hypoxia-Inducible Factor 1-alpha (HIF-1a), leading to transcription of its downstream targets. Many of these downstream targets are part of mechanisms to survive under these hypoxic conditions. These include for example proteins involved in glycolysis, cell cycle regulation, angiogenesis and hemopoiesis.[35]

Measuring tumor hypoxia

As hypoxia leads to treatment resistance, there is a need to determine the hypoxic status of a tumor. Measuring hypoxia in solid tumors is challenging and several methods are available. These include measurements using polarographic needles, exogenous and endogenous markers of hypoxia, hypoxic gene signatures, hypoxia-based PET-tracers and diffusion weighted MRI.

Eppendorf histography

Tissue oxygen tension may be assessed using electrode measurements.[36] A needle is placed inside the tumor and a probe is inserted through the needle to assess the pO_2 using an electrode. The needle is inserted into the tumor and retracted in increments through the tumor, creating several measurements and giving a three-dimensional overview of the pO_2

within the tumor.[37] Gatenby and colleagues performed this procedure in a cohort of 31 HNSCC patients with fixed lymph nodes and found that patients with pO_2 values below 10 mmHg were more likely to be non-responders to radiation therapy.[38] While this method seems reliable to assess both the pO_2 as well as the response to radiation treatment, the invasive nature of the procedure is not preferable for use in clinical practice and not all tumor sites are easily accessible to perform these measurements.

Exogenous hypoxia markers

When (hypoxia-induced) radiation resistance was identified, several drugs were investigated for their radiosensitizing properties.[39] These included antibacterial and antifungal drugs. The class of 2-nitroimidazole antibiotic drugs was shown to have radiosensitizing properties and these drugs were able to diffuse homogeneously through hypoxic tissue while oxygen was not. Other experiments showed that the 2-nitroimidazole drug misonidazole was bound by cells in hypoxic circumstances, specifically to thiol-containing proteins in cells, in particular when exposed to pO_2 levels below 10 mmHg.[40–43] The hypoxia marker pimonidazole may be administered to patients intravenously prior to biopsy. The presence of pimonidazole is then detected using immunohistochemistry.

Endogenous hypoxia markers

All nucleated cells in the body respond to hypoxia through a local cellular response.[44] As hypoxia induces a cellular and transcriptional response, the proteins that are increased under hypoxia may also be detected as hypoxia markers, using immunohistochemistry, immunofluorescence or alternative methods. As these proteins are endogenous to the body they are considered endogenous hypoxia markers in contrast to exogenous markers that have to be administered to patients externally. This is a great advantage of endogenous hypoxia markers especially for (retrospective) research purposes. The best known endogenous hypoxia markers are discussed.

HIF-1a

The best described cellular hypoxia response mechanism is the Hypoxia-Inducible Factor 1 (HIF-1) pathway (**Figure 6**).[45] HIF-1 is a transcription factor, existing of an HIF-1 alpha (HIF-1a) and HIF-1 beta (HIF-1b or Aryl Hydrocarbon Receptor Nuclear Translocator or ARNT) subunit. The cellular concentration of HIF-1a is oxygen dependent; it is constitutively expressed, but under normoxic circumstances it is quickly degraded by prolyl hydroxylases 1-3 (mostly PHD2) and further ubiquitinated by the Von Hippel-Lindau (VHL) protein.[46] The hydroxylation process is O_2 dependent and therefore degradation of HIF-1a is reduced under hypoxic circumstances.

As a transcription factor, HIF-1 attaches to hypoxia-response elements (HREs) in the DNA leading to increased transcription of its downstream targets. This HRE is characterized by

the pattern of RCGTG as a constant factor of the binding site.[47] Moreover, multiple genes are downregulated in a HIF-dependent manner without binding of HIF to these genes.[48] HIF-1a may be detected using immunostaining and is therefore considered an endogenous marker of hypoxia.

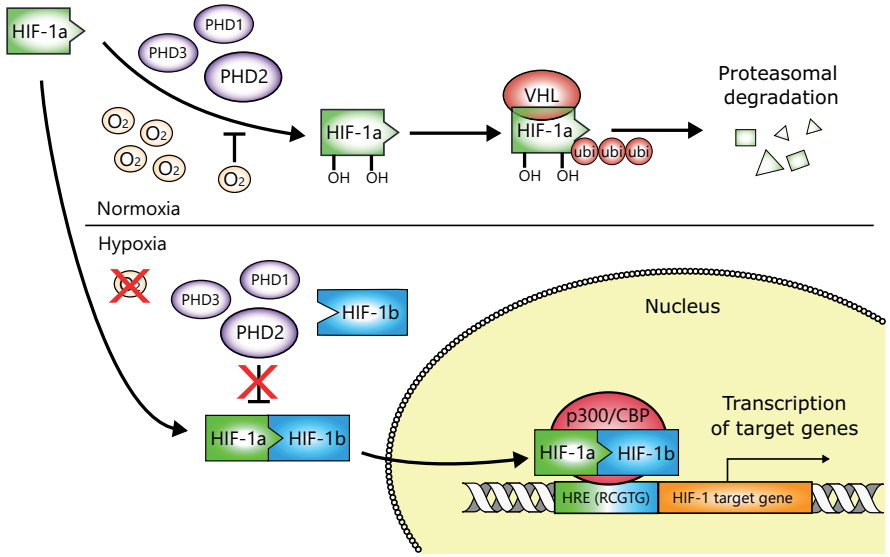

Figure 6 | Regulatory mechanism of HIF-1a. HIF-1a is constitutively expressed, but is hydroxylated and ubiquitinated under normoxic circumstances (top) by prolyl-hydroxylases 1-3 (mostly 2) and VHL, respectively, and degraded. Because this process is oxygen-dependent, under hypoxia (bottom) HIF-1a accumulates and binds to HIF-1b. Together with transcription co-activators such as p300/CBP this HIF-1 complex induces transcription of its target genes.

While HIF-1a accumulation under hypoxia has well been established, there is debate on the specificity of HIF-1a accumulation for hypoxia. Several studies have shown only weak correlations of HIF-1a to pimonidazole or Eppendorf pO_2 histography.[49–51] Moreover, intracellular HIF-1a concentrations may also increase as a result of oncogene gain or tumor suppressor gene loss of function.[52] As HIF-1a is a transcription factor, immunohistochemical staining is observed mainly in nuclear cellular compartments. Staining in other compartments such as the cytoplasm and membranous HIF-1a staining have been observed and used in studies as a hypoxia marker. However, the biological relevance of such staining is debatable.

The effect of HIF-1a accumulation on clinical outcome has been established in several studies in HNSCC.[22] Interestingly, these studies report several different staining patterns for

HIF-1a: perinecrotic staining patterns, as well as diffuse staining patterns.[53–55] In clear cell renal cell carcinoma degradation of HIF-1a is often inhibited because of a mutation in VHL. This genetic mutation results in diffuse HIF-1a staining.[56,57] Because of this, RCC is often used as a positive control in studies with immunohistochemical analysis of HIF-1a. It is argued that perinecrotic staining patterns of HIF-1a may be an indication of tumor hypoxia, while diffuse staining can be induced by oncogenic activation. A perinecrotic staining pattern was associated with poor patient outcomes compared to diffuse HIF-1a staining patterns in a study in breast cancer.[53] Interestingly, similar results were not found in a cohort of patients with radiotherapy-treated OPSCC.[55]

CA-IX

Hypoxic circumstances lead to acidic environments, based on the lack of oxygen and a switch to a glycolytic metabolism. Regulation of the acidity is crucial to cellular survival. Carbonic Anhydrases (CAs), specifically the subtype CA-IX, is a membrane protein involved in regulation of acidity under hypoxia.[58] It hydrates pericellular CO_2 to bicarbonate ions and protons. These bicarbonate ions are then actively transported inward through bicarbonate transporters to neutralize intracellular protons.

It has been shown that CA-IX (and to a certain extent also CA-XII) is regulated by hypoxia and that it colocalizes with pimonidazole in some skin and bladder carcinomas.[59] In a study in HNSCC this colocalization to pimonidazole expression could not be confirmed.[60] Therefore the specificity of CA-IX for hypoxia may also be debated. A positive correlation with HIF-1a staining is not always observed. A possible explanation is that CA-IX expression may be a better reflection of the transcriptional activity of HIF-1a, rather than hypoxic HIF-1a accumulation.

GLUT-1

Another downstream target of HIF-1a is the glucose transporter 1 (GLUT-1) protein. As mentioned above, the glycolytic metabolism of hypoxic cells increases the need for glucose. HIF-1a increases GLUT-1 and GLUT-3 transcription. Some studies show a correlation between pimonidazole staining and GLUT-1, while others do not.[60,61]

Osteopontin

Osteopontin (OPN) is a protein that is upregulated under hypoxia through a HIF-independent pathway. Overexpression of OPN involves a *Ras*-activated enhancer and is dependent on *Akt*.[62] Interestingly, OPN has also been shown to increase intracellular HIF-1a through a *PI3K-Akt* related pathway.[63] OPN overexpression in tissue is observed in cytoplasm and also extracellularly secreted.[64] In fact, plasma OPN levels are also often investigated as hypoxia markers as an alternative to immunohistochemical staining in tissue.[65]

Osteopontin overexpression leads to increased cellular survival, invasion and angiogenesis, which are all properties that are favorable under hypoxic circumstances. Moreover, OPN overexpression is associated with distant metastasis in various tumor types.[66]

A note on endogenous and exogenous markers

While ideally a marker indicates the presence or absence of a certain trait, identifying a single marker for hypoxia is challenging.[67] The co-existence of acute, chronic and transient hypoxia within the same tumor is one of the major challenges for the identification of such a marker. While pimonidazole is considered a 'gold standard' in many (preclinical) studies, it is of note that pimonidazole binding occurs only after 30 minutes after injection and there is a plateau phase after 6-8 hours in pimonidazole binding.[28,68] Moreover, the administration of pimonidazole to patients in a clinical setting in a set time before taking a biopsy may be logistically challenging.

To identify transient hypoxia, one study investigated xenograft models where mice were injected with pimonidazole every hour for 8 hours ($t_{1/2}$ of pimonidazole in mice: 30 minutes).[28] In essence, the goal was to 'saturate' these mice in pimonidazole to ensure that all hypoxic regions were stained. One hour after the final pimonidazole injection, an injection with another hypoxia marker of the 2-nitroimidazole class (CCI-103F) was administered. There were some tumor cells labeled positive for CCI-103F while negative for pimonidazole. This suggests that the cells were not hypoxic during the 8 hour period of pimonidazole labeling but were hypoxic at the time of CCI-103F labeling. This indicates the presence of areas of transient hypoxia within a tumor.

In contrast, HIF-1a concentrations may rise as fast as only two minutes under hypoxia, and may decrease again after 30 minutes of re-oxygenation.[69] For pimonidazole binding, the required time of hypoxia is longer and the binding is permanent and therefore maintained even after reoxygenation.[70] CA-IX expression will occur even later than HIF-1a accumulation, as it requires the step of transcription under the influence of HIF-1a.[71] Even after re-oxygenation and degradation of HIF-1a, CA-IX may still be expressed by the tumor. CA-IX is therefore a sign of HIF-1a's transcriptional activity, rather than its accumulation or overexpression. This explains that there may be a poor correlation between HIF-1a and CA-IX expression.[60]

Therefore, the challenges in all markers for hypoxia, both endogenous and exogenous, is not only their sensitivity and specificity for hypoxia, but the fact that they may be visualized after different durations of hypoxia. The presence of transient hypoxic areas further complicates this issue. It is therefore necessary to return to the clinical question: which hypoxia markers are able to distinguish patients with a good and a poor prognosis and which hypoxia markers aid in the selection of hypoxia-modified treatment adaptations.

Hypoxia gene signatures

Alternative to investigating hypoxia on a protein-based level by the use of biomarkers, several hypoxia gene-signatures have been investigated for their ability to classify hypoxic versus non-hypoxic tumors.[72] They are based on an altered, hypoxia-induced, transcriptional response not only of HIF-1a target genes but also others. In these studies, gene selection is performed *in vitro* in tumor cell lines. The gene expression patterns of cell lines cultured under normoxic and hypoxic circumstances are compared. Genes are then included or excluded from the final 'classifier' gene signature for their effect on patient prognosis.[73,74] While such classifiers carry prognostic value, it does not automatically translate to a classifier able to predict the response to hypoxia modification of treatment.

In one study, not only the hypoxic status, but the pH was altered in such an *in vitro* experiment, as some hypoxia-regulated genes may also be regulated by pH.[75,76] This hypoxia gene-signature has also been shown to have predictive value for the benefit from hypoxic modification of therapy in the form of nimorazole addition to radiotherapy. Another gene-signature is predictive of the benefit from hypoxic modification of radiotherapy by accelerated radiotherapy, carbogen and nicotinamide (ARCON) therapy in laryngeal cancer patients.[77]

PET-CT

Positron-emission tomography and computed tomography (PET-CT) is an imaging modality used to visualize certain tissue characteristics. The most commonly used PET-tracer is 2-deoxy-2-[^{18}F]fluoro-D-glucose ([^{18}F]FDG). Although expression and/or activity levels of GLUT1 contribute to the pattern and intensity of [^{18}F]-FDG expression, PET-CT using this tracer cannot predict expression of clinically relevant histopathological hypoxia biomarkers, e.g. HIF-1a, in HNSCC.[78]

The use of specific hypoxia tracers is an alternative way to determine tumor hypoxia *in vivo*. One benefit of the use of hypoxia-PET-CT is that it provides an overview of the hypoxic status throughout a whole tumor, rather than only on a single biopsy and thus only a part of the tumor. This is important particularly because of heterogeneity in oxygen levels within a single tumor that may be present.

There are currently several specific hypoxia PET-tracers available.[79,80] A number of these tracers are from the same 2-nitroimidazole class as the exogenous hypoxia marker pimonidazole, such as [^{18}F]-FMISO, [^{18}F]-FAZA and third-generation 2-nitroimidazole drug [^{18}F]-HX4. Uptake of these markers in relation to hypoxia has been shown to be reasonable, however there are some issues with PET-CT based hypoxia detection. One of these issues is the spatial resolution: the voxel size of modern clinical PET scanners is around 4-6 mm, while hypoxia may occur on length scales of around 100 μm.[80] Also, uptake of these tracers

is limited to vital cells and is not observed in areas of necrosis. Therefore, areas of necrosis may influence tracer uptake and may hinder an accurate visualization of hypoxic regions.

Despite these shortcomings, there is some (albeit limited) evidence of clinical relevance. It has been demonstrated that high uptake of hypoxia-PET tracers is associated with poor survival in HNSCC.[81–84] The study by Rischin *et al* described that hypoxia in [^{18}F]-MISO-PET was a predictive biomarker for response to the hypoxic cytotoxin tirapazamine.[85] Other studies on hypoxia PET-CT for response prediction to hypoxia-modified treatments are currently not available.

Diffusion-weighted MRI

Diffusion-weighted magnetic resonance imaging (DW-MRI) is an MRI sequence that relies on the diffusion of water particles within the tissue.[86] By acquiring at least two images with different motion probing gradients, i.e. two different *b*-values, the apparent diffusion coefficient (ADC) is provided. The ADC is relevant to determine tissue characteristics: a low ADC, corresponding to restricted movement of water particles, is associated with high cellularity and higher nuclear-cytoplasmic ratios.[87]

Because the DW-MRI describes the microstructure of a tumor, it may also be correlated to hypoxia: hypoxic areas may contain more necrosis, causing less restricted diffusion of water particles. Interestingly, a study comparing ADC to [^{18}F]-MISO uptake showed a lower ADC in hypoxic subvolumes, compared to non-hypoxic subvolumes.[88] Another study compared hypoxia determined in biopsies using Blimp-3, a HIF-1a transcription target, as a hypoxia biomarker and also found lower ADCs in more hypoxic pancreatic carcinomas.[89] This was also confirmed in a study that investigated ADC versus pimonidazole staining in whole-mount sections of prostate cancer.[90] The authors assume that the presence of chronic hypoxia leads to more aggressive phenotypes and therefore higher cellularity. The latter was also observed in a preclinical animal study.[91]

As the ADC reflects the tissue microarchitecture, which is composed of many different variables including cellularity and other features of the tumor microenvironment, such as hypoxia, the relation between these two parameters should be further explored.

Hypoxia and immunity in the tumor microenvironment

The transcriptional activity of HIF-1a promotes a beneficial environment for cellular survival. An acidic tumor environment and a glycolytic metabolism make an hypoxic microenvironment an excellent environment for survival and thriving of tumor cells. The hypoxic environment also influences the antitumor immune response. This occurs because of a two-fold mechanism: the hypoxic cellular response of tumor cells results in the release of immunosuppressing cytokines and the lack of oxygen itself also causes HIF-1a stabilization in immune cells and adapts their response.

Effects of HIF-1a stabilization in immune cells

HIF-1a expression occurs in immune cells through oxygen-dependent and oxygen-independent pathways and has both immunosuppressive and pro-immunogenic properties at the same time.[92,93] Activation of toll-like-receptors in myeloid cells or T-cell receptors in T-cells lead to increased HIF-1a synthesis through *NF-κB* and *PI3K* related pathways respectively. For T-cells, the activation of HIF-1a is beneficial when activated, as activated T-cells rely more on glycolytic metabolism than naïve T-cells. In contrast, HIF-1a activation in hypoxic microenvironments also increases shift of naïve T-cells to regulatory T-cells, which will have an immunosuppressing effect. Finally, while expression of HIF-1a increases the lytic capabilities of cytotoxic (CD8+) T-cells, it also delays the T-cell differentiation into CD8+ T-cells.[94,95]

Immunomodulation by tumor cells

Hypoxic tumor cells are also able to attract macrophages (tumor-associated macrophages or TAMs). The hypoxic microenvironment shifts TAMs from an activated M1 phenotype toward an M2 phenotype that suppresses the tumor-immune reactions, causing tumors to evade immune reactions.[94,96] Moreover, TAMs increase endothelial and malignant cell proliferation, increase survival under chemotherapy and promote metastasis.[97–99]

Another transcription target of HIF-1a is the programmed death-ligand 1 (PD-L1), which is part of an immune checkpoint together with the programmed death-receptor 1 (PD-1) on immune cells.[100] Increased upregulation of PD-L1 by tumor cells causes inhibition of the antitumor immune response. Immune therapy that blocks the PD-1/PD-L1 binding through antibodies directed against either of these proteins is currently being investigated and used in clinical practice in many solid tumors including HNSCC.[101] It has recently been shown that PD-L1 may also be regulated under hypoxia through HIF-1a, causing immune evasion by inhibiting killing by cytotoxic T-cells or natural-killer T-cells.[102]

In summary, HIF-1a is upregulated under hypoxic circumstances in immune cells, which has both immunosuppressive and pro-immunogenic effects. However, tumor cells also gain immunosuppressive properties under hypoxia that suppress the immune system and prevent tumor cell killing.

Overcoming hypoxia: treatments targeting hypoxia or HIF-1a

Several strategies have been investigated to overcome tumor hypoxia and increase treatment susceptibility of hypoxic tumors.[103] While a review has shown a statistically significant improvement in patient outcome, hypoxia-modified treatments are currently not a part of clinical routine.[104] Efforts to improve tumor oxygenation started with application of normobaric oxygen and later hyperbaric oxygen. However, this was abandoned as it was

difficult to apply logistically in clinical care and a review has shown insufficient evidence of effect.[104] Currently other approaches are being investigated.

ARCON

The concurrent application of radiotherapy in combination with breathing of carbogen is termed ARCON and is a method to increase tumor oxygenation.[105] The breathing of carbogen gas, a mixture of 2% CO_2 and 98% oxygen, increases oxygenation not only by increased oxygen supply, but also by increasing the respiratory drive and vasodilation because of the CO_2. Nicotinamide is an amide derivative of vitamin B_3 and has radiosensitizing properties, but also decreases intermittent vascular shutdown and therefore decreases perfusion-limited hypoxia.[105,106] A randomized trial in stage II-IV LSCC patients of accelerated radiotherapy (AR) versus ARCON has shown an overall benefit in regional control, but not local control or overall survival. [107]

Radiosensitizer application

The best described radiosensitizing drug as a hypoxia-radiosensitizer is nimorazole, a drug of the 2-nitroimidazole class.[108] Drugs of the 2-nitroimidazole class mimic oxygen under hypoxic circumstances, thus enabling the formation of DNA strand breaks during radiotherapy (**Figure 7**).[109] While it has been shown to be effective in a trial in Denmark, an international trial on its effectiveness was shut down early because of recruitment issues.[110]

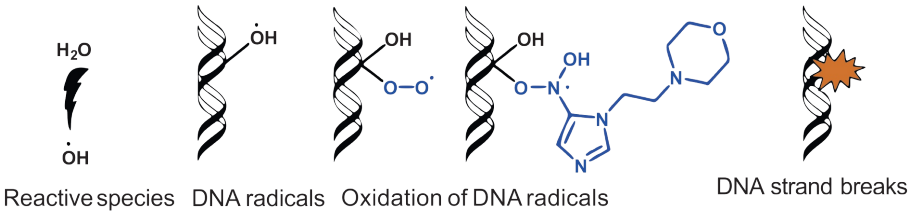

Figure 7 | Example of oxygen-mimicking. Radiation induces reactive oxygen species that bind to the DNA. In order for DNA strand breaks to occur, oxidation of these DNA radicals is required. This may be achieved by binding oxygen or an oxygen mimicking molecule, such as nimorazole (shown in blue). *Imaged from Jackson et al.*[109]

Other strategies to overcome hypoxia and HIFs

Other current efforts include the application of hypoxia-activated pro-drugs, and drugs that improve the efficiency of the vascularity using angiogenesis-inhibiting agents of vascular disrupting agents.[111] Although these agents disrupt tumor vascularity, they are thought to

also improve vascularity by primarily targeting the inefficient vessels involved in areas of perfusion limited hypoxia as shown in **Figure 5**.[112] Another strategy to overcome the effects of hypoxia is by direct inhibition of HIF-1a. Several drugs are currently being investigated in phase II or III trials.[27,113]

Conclusion

In conclusion, hypoxia is a well-known adverse micro-environment factor in a tumor's response to therapy, tumor aggressiveness and metastasis. Moreover, there is an interaction between hypoxia and other factors within the tumor microenvironment including immunity. There are existing and promising new therapies that may reduce tumor hypoxia or specifically target hypoxic tumor cells, and these therapies have greater effect when patients with hypoxic tumors are preselected. Therefore there is a need to further investigate the role of hypoxia and the role of HIFs in HNSCC, in particular in relation to the tumor microenvironment, and to find a reliable biomarker to determine the hypoxic status of a tumor to assess eligibility for hypoxia-modified or targeted treatment.

Aim of this thesis

This thesis focuses on the role of hypoxia and more specifically HIF-1a in relation to the tumor microenvironment and patient outcomes in HNSCC. In **Chapter 2** we investigate the role of HIF-1a as a hypoxia marker in correlation to pimonidazole as a reference standard.

In **Chapter 3** we compare the expression of HIF-1a as a hypoxia marker to PD-L1 expression and tumor-infiltrating lymphocytes, as two other factors within the tumor microenvironment in an OPSCC patient cohort. In **Chapter 4** we compare tumor and tumor microenvironment factors to imaging features in DW-MRI to see whether this modality is able to determine the hypoxic status in OPSCC patients.

In the following chapters, we investigate the effect of hypoxia on outcomes in HNSCC patients. In **Chapter 5** we investigate the literature for the clinical relevance of endogenous biomarker expression for patients treated with regular (non-hypoxia modified) therapy. In **Chapter 6** we investigate the role of HIF-1a overexpression in patients with HPV-positive versus HPV-negative OPSCC. In **Chapter 7** we compare HIF-1a expression and clinical outcome in patients with tumors from different sites in the head and neck. **Chapter 8** summarizes the findings of these studies and provides a view on future perspectives.

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Image accountability

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Chapter 2

Correlation and colocalization of HIF-1α and pimonidazole staining for hypoxia in laryngeal squamous cell carcinomas: A digital, single-cell-based analysis

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Abstract

Objective: Tumor hypoxia results in worse local control and patient survival. We performed a digital, single-cell-based analysis to compare two biomarkers for hypoxia (hypoxia-inducible factor 1-alpha [HIF-1α] and pimonidazole [PIMO]) and their effect on outcome in laryngeal cancer patients treated with accelerated radiotherapy with or without carbogen breathing and nicotinamide (AR versus ARCON).

Materials and Methods: Immunohistochemical staining was performed for HIF-1α and PIMO in consecutive sections of 44 laryngeal cancer patients randomized between AR and ARCON. HIF-1α expression and PIMO-binding were correlated using digital image analysis in QuPath. High-density areas for each biomarker were automatically annotated and staining overlap was analyzed. Kaplan-Meier survival analyses for local control, regional control and disease-free survival were performed to predict a response benefit of ARCON over AR alone for each biomarker.

Results: 106 Tissue fragments of 44 patients were analyzed. A weak, significant positive correlation was observed between HIF-1α and PIMO positivity on fragment level, but not on patient level. A moderate strength correlation ($r = 0.705$, $p < 0.001$) was observed between the number of high-density staining areas for both biomarkers. Staining overlap was poor. HIF-1α expression, PIMO-binding or a combination could not predict a response benefit of ARCON over AR.

Conclusion: Digital image analysis to compare positive cell fractions and staining overlap between two hypoxia biomarkers using open-source software is feasible. Our results highlight that there are distinct differences between HIF-1α and PIMO as hypoxia biomarkers and therefore suggest co-existence of different forms of hypoxia within a single tumor.

Introduction

Tumor hypoxia results in worse local control and patient survival in patients with cancer, including those with head and neck squamous cell carcinoma (HNSCC).[1–3] The best method to classify a tumor as hypoxic or normoxic is still a matter of debate. It may be done in both tissue and imaging-based techniques, including but not limited to Eppendorf pO_2 histography,[4–6] [^{18}F]-MISO or [^{18}F]-AZA PET,[7] or BOLD- or DW-MRI.[8,9] Tissue-based techniques include the use of biomarkers for hypoxia. These biomarkers may be detected in biopsies taken as a part of routine clinical work-up.

While multiple hypoxia biomarkers are available, each marker has distinct differences and an 'ideal hypoxia biomarker' is not available. Two types of biomarkers are distinguished: endogenous and exogenous biomarkers. Hypoxia will trigger a cellular response to improve cellular survival under hypoxic circumstances.[10] Proteins upregulated under hypoxia may be used as endogenous biomarkers for hypoxia. One of the best described endogenous hypoxia biomarkers is the transcription factor Hypoxia-inducible factor 1-alpha (HIF-1α).[11] This protein is constitutively expressed, but is quickly degraded under normoxic circumstances. Under hypoxic circumstances HIF-1α accumulates in cells and together with the HIF-1β subunit this transcription factor binds to hypoxia-responsive elements (HREs) on the DNA to upregulate cellular survival mechisms.[12] Downstream targets of HIF-1α include carbonic anhydrase IX (CA-IX) and glucose-transporter-1 (GLUT-1), which are also considered biomarkers for hypoxia. For all these markers, it has been shown that high expression is related to worse outcome in HNSCC patients.[2]

Exogenous hypoxia biomarkers are drugs that are administered to patients prior to biopsy. Hypoxia-activated pro-drugs are a subgroup of exogenous hypoxia biomarkers that are activated when tissue pO_2 reaches below a certain threshold.[13] These drugs are selectively metabolized or bound by hypoxic cells.[14] Pimonidazole (PIMO), a drug of the 2-nitroimidazole class of antibiotics, is irreversibly bound to proteins in the cytoplasm below pO_2 levels of 10 mmHg. After administering PIMO to patients intravenously before biopsy, PIMO binding may be detected using immunohistochemistry.[15]

Radiotherapy is an important treatment modality in HNSCC, but its effectivity is reduced under hypoxia.[16] Therefore, treatment modifications have been developed to improve tumors' sensitivity. One of these modifications is accelerated radiotherapy (AR) with carbogen breathing and nicotinamide (ARCON). Carbogen (a gas mixture of 98% O_2 and 2% CO_2) and nicotinamide (a vasoactive agent) improve tumor oxygenation. Therefore, ARCON is thought to increase the sensitivity to radiotherapy.[17] A trial in laryngeal squamous cell carcinoma (LSCC) patients has shown improved regional control and a trend toward improved disease-free survival in patients treated with ARCON compared to patients treated with AR alone in tumors with high PIMO binding.[18] As there are distinct differences

between HIF-1α and PIMO as hypoxia markers, it raises the question how HIF-1α and PIMO correlate and whether HIF-1α alone or a combination of PIMO and HIF-1α is better able to identify patients that benefit from the addition of ARCON over AR alone.

The comparison of biomarker expression may be done using visual scoring by a pathologist.[19] This is currently seen as the reference standard, even though this method may suffer considerable inter-observer variability depending on the staining.[20,21] Alternatively, digital image analysis has been performed for hypoxia markers in several studies.[22,23] These studies compared both markers based on the number of positive pixels or positive staining area. Although this method is more objective than visual scoring, it does not take into account cellular features such as size, nor whether the staining was present in the nucleus or cytoplasm. QuPath is a free, open-source software package that performs biomarker analysis by identifying biomarker expression in individual cells.[24] Moreover, it detects staining positivity in the nuclear and cytoplasmic cell compartments separately so that only the biologically relevant cellular compartment is considered.

The goal of the present study was to compare HIF-1α and PIMO expression in LSCC patients participating in a randomized controlled trial of ARCON versus AR. This was done using a digital image analysis using QuPath where we compared not only positive cell counts, but also the location of their expression within the tissue and staining overlap. Finally, the effect of HIF-1α and PIMO staining on survival and the benefit of ARCON over AR for tumors positive for these hypoxia biomarkers were investigated.

Patients and methods

Patient cohort

Laryngeal tumor biopsies from 58 patients who participated in a phase III randomized trial were used in this study (**Figure 1**).[18] In this trial 345 patients with LSCC were randomized to AR or ARCON. Inclusion criteria were classification T2b and higher, any N-stage, no distant metastases, WHO performance status 0 or 1, age above 18 years and written informed consent. Institutional review board approval was obtained from the Radboud University Centre Nijmegen (Radboudumc) Medical Research Ethics Committee. Seventy-nine of these patients participated in a translational side study and had received PIMO intravenously two hours before biopsy. The 58 patients in the present study were a subgroup of these 79 patients that had sufficient tissue available in the pathology archives of the Radboudumc.

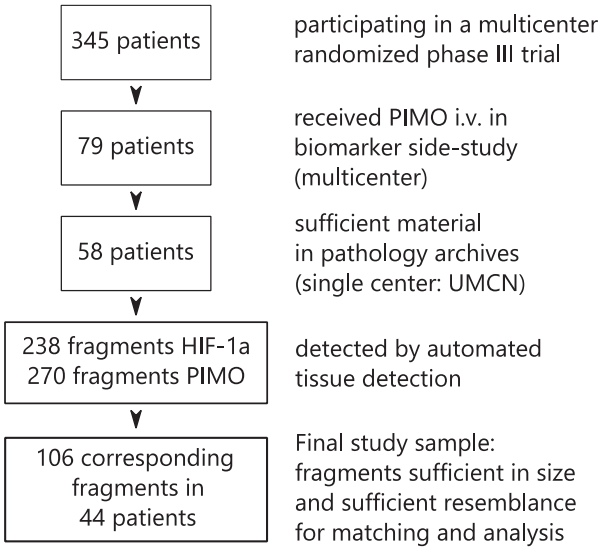

Figure 1 | Patient inclusion flowchart.

Immunohistochemical staining

Consecutive tissue sections were used for HIF-1α and PIMO-staining. For PIMO staining, sections were deparaffinized, followed by rehydration using Histosafe (Pathosafe, Selargius, Italy). Antigen retrieval was performed using a citrate buffer for 30 min. Endogenous peroxidase activity was blocked using a 3% H2O2 buffer in methanol. Sections were pre-incubated with Primary Antibody Diluent (PAD, Cat# BUF014, Bio-Rad, Veenendaal, the Netherlands) and blocked with normal donkey serum 5% in PAD. The primary antibody was a Mouse-anti-PIMO 1:50 in PAD overnight at four degrees Celsius (Lot# 9.7.11, HydroxyProbe, Massachusets USA), followed by a donkey-anti-mouse secondary antibody (Cat# 715–066-150, Jackson Immuno Research Laboratories Inc, Ely, UK). An ABC reagent was applied, followed by the DAB staining and counterstaining with hematoxylin.

Immunohistochemical staining for HIF-1α was performed as previously described.[25] In brief, sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked using a 3% H2O2 solution in PBS. Antigen retrieval was performed by boiling the slides in a pH 9.0 EDTA buffer for 20 min. The Novolink kit (Leica Biosystems, Rijswijk, the Netherlands) was used according to the manufacturer's instructions for staining. Incubation with the primary antibody (Mouse-anti-HIF-1α, BD Biosciences, cat# 610959, lot 4 073 775, diluted 1:50 in PBS-BSA) was performed overnight at four degrees Celsius. For every staining batch, a renal cell carcinoma tissue section was used as a positive and negative control by incubation with the primary antibody or PBS-BSA to ensure similarity of the staining.

Digital image analysis

All sections were digitized using a Hamamatsu Nano Zoomer XD scanner at 40x magnification. Analyses were performed using QuPath.[24] First, separate tissue fragments were automatically detected on each section and exported to separate image files (**Figure 2a, b**). The corresponding HIF- and PIMO-stained tissue fragments were manually matched and automatically aligned in ImageJ using the TrakEM2 plugin (**Figure 2c**).[26] When automatic alignment was not possible, alignment was performed manually in TrakEM2.

For each fragment, the tissue was automatically identified and annotated in QuPath. Areas of necrosis, healthy epithelium, scanning artifacts and areas of foldover were manually removed from the annotation. The overlapping area of the annotations in the corresponding HIF-1α and PIMO tissue fragments was used for analysis to ensure that the analyzed area was identical for both stains.

Cell detection was performed in QuPath using the settings in **Supplementary information S1**. (**Figure 3**). As the staining intensities of HIF-1α and PIMO were different, a different threshold was used for each stain. In most tumors HIF-1α staining was quite diffuse, although there were regions with more intense staining. Preliminary comparisons of HIF-1α and PIMO staining patterns suggested co-localization between PIMO staining and the intense regions of HIF-1α staining. To investigate this hypothesis only strong HIF-1α staining was taken into account in the following analyses. A cutoff for strong HIF-1α positivity was set by creating a composite (training) image from 17 different patients and visually identifying the best cutoff value. The researcher determining the threshold for HIF-1α positivity did not have knowledge of the PIMO expression of the corresponding areas (and vice versa) at that moment. Thus, thresholds were set in an unsupervised (blinded) manner. Cells with strong positivity for HIF-1α were defined as cells with an optical density (OD) of the DAB color > 0.65 in the nucleus. The staining intensity of PIMO was relatively weak and the cutoff for PIMO positivity was set at a DAB OD > 0.10 in the cytoplasm. Following positive cell detection, hypoxic regions were identified. This was done by automatically annotating regions with a high density of positive cells using a publicly available script.[27] The parameters used for hypoxic region detection were visually optimized. Identical settings were used for HIF-1α and PIMO and are provided in Supplementary information S1.

To convert the values from fragment to patient level, the total number of positive and negative cells for all tissue fragments within one patient were summed. On patient level, tumors with 2.6% or more PIMO positive cells were considered hypoxic, in concurrence with the original study.[18] For strong HIF-staining (with weak or moderate staining not taken into account) no staining cutoff is available in previous literature. Therefore, the median value of 6.2% strongly HIF-positive cells was used as a cutoff to classify a tumor as hypoxic.

Concurrence in classification as normoxic versus hypoxic tumors between both hypoxia markers was investigated by Pearson correlation analysis and the McNemar test. Statistical significance was set at $p \leq 0.05$. Overlap scores of the two stains were calculated using Dice-scores, positive predictive value (PPV) and conformity index (C-index). The Dice score describes the relationship of the overlapping area in relation to the total area.[28] It is calculated as Dice = 2 * overlapping area / (area of HIF-1α staining + area of PIMO staining).

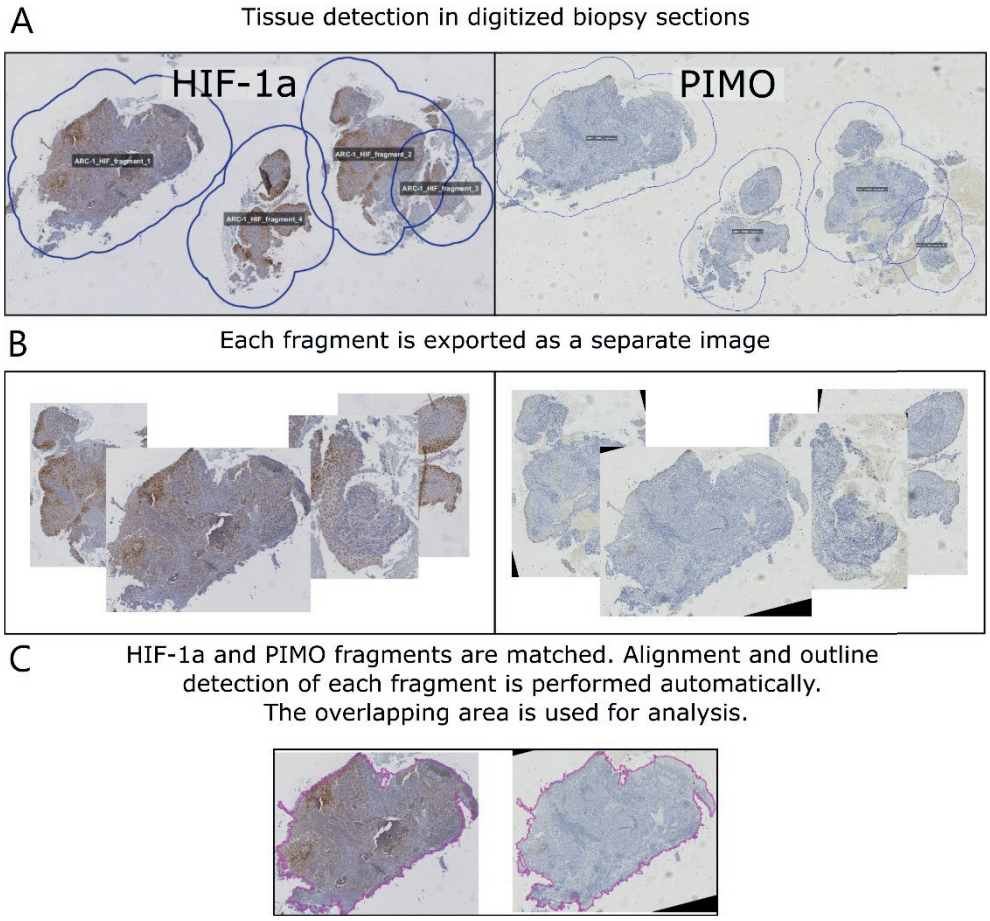

Figure 2 | Image preparation process. A shows a scanned tissue section containing four separate tissue fragments for HIF-1α and PIMO. Using the 'Thresholding' function in QuPath the tissue fragments were automatically detected. Each tissue fragment is exported to a separate file as shown in B. C shows matching fragments of HIF-1α (left) and PIMO staining (right). The tissue outline of each fragment was automatically detected and the overlapping area of both fragments (shown in pink) was used for the analyses. Analyses were performed on a per fragment level (i.e. for each corresponding fragment individually) and on a per patient level (i.e. by summing the data of the individual fragments belonging to each patient).

The C-index also describes the relationship of the overlapping area to the total area as does the Dice-score, albeit slightly different, and is often used to compare radiotherapy plans.[29] It is calculated as C-index = overlapping area / (area positive for HIF-1α + area positive for PIMO – overlapping area)). The PPV is a measure of how likely a positive score of a certain test relates to a positive score using the reference standard. In the present study the PPV was calculated with HIF-1α as the test and PIMO as the reference standard. It was calculated as PPV = true positive area / (true positive + false positive area).

Survival analyses

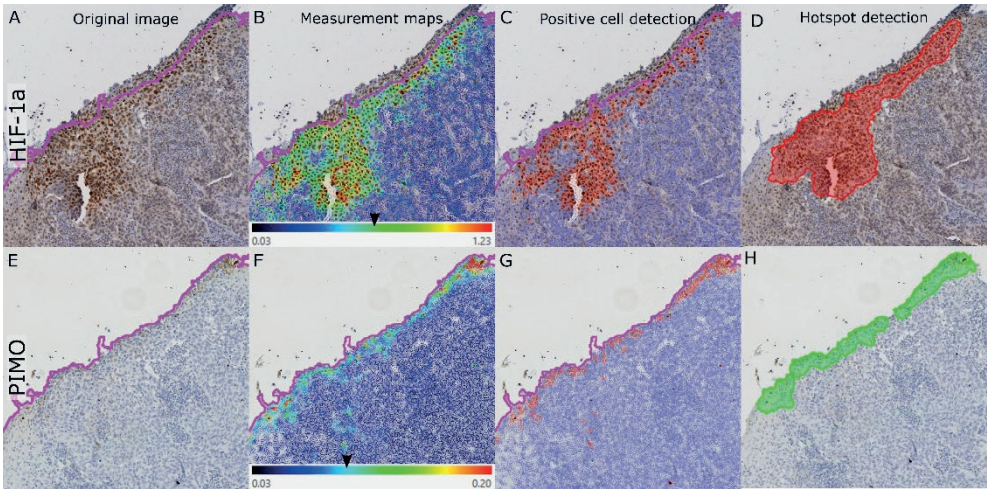

Figure 3 | Positive cell detection. A representative area of a biopsy is shown. Note that PIMO staining was less intense than HIF-1α staining. Each row depicts the original image with the tissue annotation (A and E), the measurement map with the corresponding legend (B and F), followed by the final positive cell detection with threshold 0.65 optical density for HIF-1α and threshold 0.10 for PIMO marked with an arrow (C and G). Here blue cells are negative and red cells are positive. The automatically annotated hotspots are shown in D and H.

Of each patient, the date and type of local recurrence, regional recurrence or the occurrence of distant metastases as well as the date and reason of death and date of last follow-up visit were recorded. Follow-up was at least 5 years from the date of randomization for all surviving patients. For local control (LC) and regional control (RC), the incidence of such a recurrence was considered an event and patients were censored at the moment of the last follow-up or death. For disease-free survival (DFS), the date of any recurrence (local, regional or distant) or death were considered an event and patients were censored at the

moment of last follow-up. The outcomes were compared using the log-rank test of a Kaplan-Meier survival analysis. All statistics were performed in SPSS Statistics version 26 (IBM).

Results

Patient characteristics

Biopsies of 58 patients were stained for HIF-1α and PIMO (**Figure 1**). After staining, digitization of the sections, and tissue fragment detection, 238 tissue fragments of HIF-1α and 270 tissue fragments of PIMO were identified, with an average of 4.25 fragments per patient. The final analysis was performed in the 106 adequately matched pairs of tissue fragments of 44 patients that were sufficient in size and contained sufficient amounts of tumor tissue (mean 2.33 fragments, range 1–9 fragments per patient). The baseline characteristics of these patients are shown in **Table 1**.

Correlations between HIF-1α and pimonidazole positivity

On a per fragment level, the median percentage of positive cells was 4.1% (IQR 1.0 – 13.7) for strong HIF-1a staining and 2.6% (0.4 – 10.9) for PIMO. The correlation between these percentages was significant but weak ($r = 0.365$, $p < 0.001$, Figure 4). On a per patient level, the median percentage of positive cells was 6.2% (IQR: 2.4 – 12) for strong HIF-1α staining and 5.1% (IQR: 0.6 – 11) for PIMO staining. The correlation between these percentages was not statistically significant ($r = 0.176$, $p = 0.253$).

Of the 106 tissue fragments, there were 45 (42.5%) with 6.2% or more HIF-1α positive cells and 53 (50%) with 2.6% or more PIMO-positive cells (**Table 2**). The McNemar test shows that these positivity rates are comparable ($p = 0.253$). The concurrence rate (HIF+/PIMO + or HIF-/PIMO-) was 62.3%. On a per patient level, there were 22 patients (50%) with 6.2% or more HIF-1α positive cells and 29 (65.9%) patients with 2.6% or more PIMO positive cells. The McNemar test shows that these positivity rates are also comparable ($p = 0.167$), the concurrence rate was 56.8%.

Staining hotspot and overlap analysis

In the previous analysis, tumors or fragments were considered hypoxic when the percentage of positive cells was above a set threshold. In the following analysis, we defined tumors or fragments positive when hypoxic regions could be detected by QuPath. In the 106 tissue fragments, hypoxic regions of strong HIF-1α staining were detected in 84 fragments (77.8%)

Variable	AR, n = 20	ARCON, n = 24
Mean age (SD)	61 (8.0)	64.6 (8.5)
Sex		
Male	14 (70)	18 (75)
Female	6 (30)	6 (25)
WHO performance status		
0	13 (65)	19 (79)
1	7 (35)	5 (21)
Site		
Glottic	5 (25)	6 (25)
Supraglottic	15 (75)	18 (75)
T-classification		
T2	6 (30)	3 (13)
T3	9 (45)	18 (75)
T4	5 (25)	3 (13)
N-classification		
N0	6 (30)	9 (38)
N1	7 (35)	3 (13)
N2a	1 (5)	1 (4)
N2b	1 (5)	2 (8)
N2c	5 (25)	9 (38)
N3	0 (0)	0 (0)
Outcomes		
Local recurrence	0 (0)	4 (17)
Regional recurrence	4 (20)	1 (4)
Distant metastasis	4 (20)	1 (4)
All-cause mortality	6 (30)	8 (33)
Events DFS	6 (30)	9 (38)

Table 1 | Baseline patient characteristics. Characteristics shown as n (%), unless stated otherwise. Abbreviations: AR = accelerated radiotherapy, ARCON = accelerated radiotherapy with carbogen breathing and nicotinamide, DFS = disease-free survival, WHO = World Health Organization.

and hypoxic regions of PIMO staining were detected in 72 fragments (68%). On a per fragment level, the concurrence rate (HIF+/PIMO + or HIF-/PIMO-) was 77.3%. A median of 3 (IQR 1 – 8.5) hypoxic regions were detected for HIF-1α and a median of 2.5 (IQR: 0 – 7) hypoxic regions were detected for PIMO. When summed to a per patient level, a median of 11 (IQR: 3.3 – 22.5) and 7.5 (3.3 – 11) hypoxic regions were detected for HIF-1α and PIMO respectively. The number of detected hypoxic regions for both stains significantly correlated both on a per fragment level (r = 0.511, p < 0.001) and on a per patient level (r = 0.705, p < 0.001, **Figure 4**).

	Per tissue fragment			Per patient		
Method 1: regarded as positive when positive fraction is above cutoff*						
	PIMO-negative	PIMO-positive	Total	PIMO-negative	PIMO-positive	Total
Strong HIF-negative	37 (69.8)	24 (45.3)	61 (57.5)	9 (60)	13 (44.8)	22 (50)
Strong HIF positive	16 (30.2)	29 (54.7)	45 (42.5)	6 (40)	16 (55.2)	22 (50)
Total	53 (100)	53 (100)	106 (100)	15 (100)	29 (100)	44 (100)
Method 2: regarded as positive when hotspots of each staining are detected						
	PIMO-negative	PIMO-positive	Total	PIMO-negative	PIMO-positive	Total
Strong HIF-negative	16 (47.1)	6 (8.3)	22 (20.8)	2 (33.3)	2 (5.3)	4 (9.1)
Strong HIF positive	18 (52.9)	66 (91.7)	84 (79.2)	4 (66.7)	36 (94.7)	40 (90.9)
Total	34 (100)	72 (100)	106 (100)	6 (100)	38 (100)	44 (100)

Table 2 | Crosstabulation of strong HIF-positivity and PIMO-positivity. Values are shown as n (%). *Cutoff-value 6.2% positive cells for HIF (median value) and 2.6% for PIMO (based on previous literature)

The hypoxic region detection analysis also meant that we were able to investigate overlap between hypoxic regions according to strong HIF-1α and PIMO-staining (**Figure 5**). In 16 fragments (15%) no hotspots of either HIF-1α or PIMO were detected. In 56 fragments

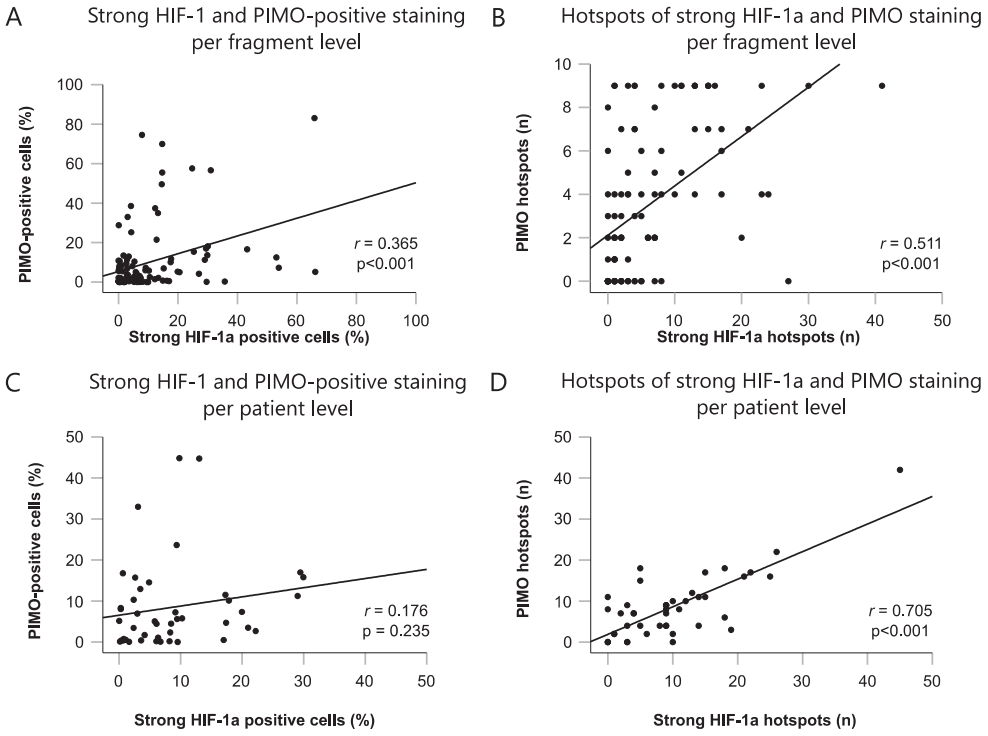

Figure 4 | Scatterplots of percentage positive cells (A, C) and number of detected staining hotspots (B, D) for strong HIF-1α and PIMO staining.

(62%) of the remaining 90 fragments, the identified hotspots of HIF-1α and PIMO showed overlap. The median Dice score was 0.01 (IQR: 0 – 0.07), the median PPV was 0.12 (IQR: 0 – 0.35) and the median C-index was 0.07 (IQR: 0 – 0.19). Interestingly, there was a large number of outliers with a high PPV. This means that in these patients the hypoxic regions according to HIF-1α staining had a high likelihood to also be PIMO-positive.

Survival analyses

The patient outcomes are shown in Table 1. In the survival analyses, high HIF-1α expression, high PIMO binding or a combination of both were not significantly associated with a difference in LC, RC or DFS ($p > 0.05$). When patients were stratified for high or low PIMO

binding, HIF expression, or positivity for one or both hypoxia markers, there was no survival difference between AR and ARCON treatment arms.

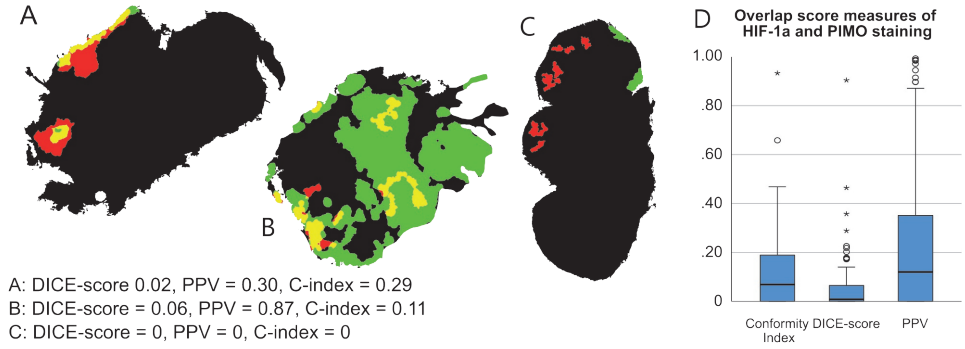

Figure 5 | Hotspot overlap analysis. Three example tissue fragments are shown with an average (A), low (B) and 0 Conformity-Index (C). Tissue outline is shown in black, HIF-1α hotspots are shown in red, PIMO hotspots are shown in green and overlapping areas are shown in yellow. These fragments are shown for illustration purposes and are not to scale. In D a boxplot is shown of the C-indices, PPV and DICE-scores of the 106 tissue fragments.

Discussion

In this digital, cell-based analysis of hypoxia in LSCC biopsies, we found a significant, but weak correlation between HIF-1α expression and PIMO binding in matching tissue fragments. This correlation was not significant when summed to a per patient level. In contrast there was a moderate correlation between the number of hypoxic regions on both a per fragment and per patient level. The spatial overlap between both stains was low. Furthermore, expression of HIF-1α, PIMO binding as well as positivity for both markers combined could not predict a benefit of ARCON over AR.

Comparison of HIF-1a and PIMO as hypoxia biomarkers

Hypoxia is an important issue in solid tumors because hypoxia increases aggressiveness, the potential for metastases, immune cell evasion and resistance to treatment.[3] While several treatment modifications are available for hypoxic tumors, the identification of hypoxic tumors for patient selection for such treatment is not a part of current clinical practice.[16,30,31] Should biomarkers be used to identify hypoxic tumors in a clinical setting, an endogenous marker such as HIF-1α would be preferable to an exogenous marker like PIMO as it would not require additional patient burden and costs.

There are distinct differences between forms of hypoxia and between hypoxia biomarkers such as HIF-1α and PIMO. For instance, PIMO is used as a hypoxia biomarker in many

preclinical and animal studies and may be considered a specific marker of hypoxia, while HIF-1α may also be upregulated through other pathways than hypoxia.[32,33] In addition, HIF-1α upregulation may be detected after minutes of hypoxia and is quickly degraded after re-oxygenation.[34] Moreover, HIF-1α requires the cell's transcriptional activity to be functional, which may be inhibited during longer periods of deeper hypoxia. In contrast, PIMO binding requires a longer duration of hypoxia below 10 mmHg pO_2.[35] And as PIMO-binding is irreversible it may be visualized both in situations where re-oxygenation has taken place and in cells that reach such critically low pO_2levels that they will soon undergo cell death or apoptosis.[36] Therefore, positivity for either hypoxia biomarkers may represent different forms of hypoxia in terms of pO_2-levels and duration.

Because of the need to administer PIMO to patients prior to biopsy, evidence on the effect of PIMO-binding on clinical patient outcome is relatively scarce. There is one study in HNSCC patients investigating the effect of PIMO binding on LRC and found a significant difference: a 2-year LRC 48% for high PIMO-binding and 87% for patients with low PIMO-binding tumors.[17] Because endogenous biomarkers can be studied more easily, and also in a retrospective manner, the body of evidence on the effect of HIF-1α on clinical outcome is larger.[2]

When comparing HIF-1α expression and PIMO-binding in our patient cohort, we observed a significant but weak correlation between HIF-1α and PIMO in separate tissue fragments on consecutive slides. Summed to a per patient level, the correlation was not statistically significant. This may originate from a lack of statistical power, as we could analyze only 44 patients in contrast to 106 tissue fragments. This discrepancy may also highlight tumor heterogeneity and an inherent difference between the two hypoxia markers; certain areas of a tumor may be more hypoxic than others, and the overall percentage or hypoxic cells may not be a good way to compare the two hypoxia markers.

Until now, manual scoring by a pathologist remains the reference standard for biomarker comparison, despite large inter-observer differences depending on the staining.[20,21] Comparison of HIF-1α and PIMO staining was previously done in 36 patients with cervical carcinoma where pathologists scored the presence of each biomarker using a visual, semi-quantitative system.[19] PIMO positivity was divided into 4 categories of percentage positive cells and HIF-1α positivity was divided into 6 categories that combined percentage positivity and staining intensity. The authors found a weak but significant correlation between these two measurements. As we performed a digital analysis, we were able to perform a more objective estimation of cell percentages and found a similar correlation when comparing corresponding tissue fragments, but not whole biopsies. Also, while we only considered strong HIF-1α positivity, the staining intensity by itself was not a part of our scoring system.

The correlation between the number of hypoxic regions on both stains was stronger than the percentage of positive cells for both stains. To our knowledge, comparison of hypoxia biomarkers in this manner has not been done previously. Therefore the (clinical) relevance of this finding is currently uncertain. We believe that this strong correlation illustrates that certain tumors have an architecture making them vulnerable to multiple forms of hypoxia, that may both be detected using HIF-1α and PIMO as hypoxia biomarkers. Possibly, the number of hypoxic regions is a better, or biologically more relevant, way than comparing positive cell fractions to compare these biomarkers. For instance, a tumor may contain small chronically deep hypoxic regions positive for PIMO surrounded by larger areas of 'mild' hypoxia positive for HIF-1α. In such cases there may be a discrepancy in positive cell percentages for both hypoxia biomarkers although two forms of hypoxia coexist.

While we identified a strong correlation between the number of hypoxic regions, the overlap between these areas was generally poor. Interestingly, we could identify subgroups with excellent and poor staining overlap. It may be possible that in patients with excellent overlap, the HIF-1α positivity was caused by hypoxia and that in the non-overlapping group HIF-1α positivity might have originated from transient hypoxia or oncogenic activation. We finally conclude that HIF-1α and PIMO may be considered complementary biomarkers for hypoxia.

HIF-1a and PIMO in relation to ARCON

In the original phase III trial of LSCC patients randomized between AR and ARCON, for patients with high PIMO positivity there was significant benefit of ARCON over AR in regional control and a trend toward better DFS with ARCON.[18] Because of the aforementioned reasons we hypothesized that tumors positive for both HIF-1α and PIMO represent tumors that are currently hypoxic but still vital. Such a subgroup could theoretically benefit most from the addition of hypoxia treatment modification or ARCON. We therefore investigated whether HIF-1α was better than or additive to PIMO in identifying patients that would benefit from the addition of carbogen breathing and nicotinamide to AR.

Unfortunately, we could not find such a role for PIMO, HIF-1α or combined positivity for these markers to predict a benefit of ARCON for LC, RC or DFS. In the original study of 79 patients, there was a significant benefit in RC in patients with high PIMO fractions.[18] In the present study, the low number of patients (44 of the 79 original patients) and events (deaths or recurrences) was a clear limitation to evaluate the predictive power for HIF-1α or a combination of HIF-1α and PIMO.

Our finding is in contrast to a previous, similar sized phase II study of patients with HNSCC treated with AR or ARCON where high PIMO fractions were associated with poor LRC and DFS in patients with HNSCC.[17] Moreover, high PIMO fractions predicted a stronger response to ARCON in this study. The number of events (recurrences and deaths) in this phase II trial

was higher than in our study and the phase III trial. Therefore the required sample size to observe a statistically significant difference between two treatment groups was much lower in this study.

Of note, the use of tissue biomarkers in diagnostic biopsies for determining hypoxic status may be hampered by tumor heterogeneity. Especially in larger tumors, a single biopsy from the superficial part of the tumor may reflect the hypoxic status of the whole tumor less well. Hypoxia biomarker analysis in excised tumors or the use of specific hypoxia (PET-) imaging techniques may provide a better overview of hypoxia in a whole tumor.[7,37] Proof-of-principle has been demonstrated for [^{18}F]-MISO-PET as a predictive biomarker for response to the hypoxic cytotoxin tirapazamine.[38] However, the optimal tissue- or imaging based predictive biomarker should be evaluated for each (hypoxia modifying) treatment specifically. In the case of tissue-based biomarkers, it remains to be further explored if a single biopsy will suffice or if multiple (deep) biopsies are required.

The use of a digital, cell-based analysis

The increase of digital pathology and free, open-source software for analysis is of great value to clinicians and researchers. Having an open-source platform is important for innovation and contribution to the software by users. Moreover, free software can also be used in resource-limited settings. QuPath offers a (positive) cell detection function, where cells are detected based on shape features for hematoxylin-DAB or immunofluorescence stained sections.[24] The DAB-staining intensity may then be determined separately in the nuclear and cytoplasmic cell compartments to classify a cell as positive or negative. This is a clear improvement over previous studies on hypoxia markers where the hypoxic fraction was determined by dividing the sum of positive pixels (positive tissue area) by the sum of total pixels (total tissue area) in immunofluorescence images.[22,23] This method does not take the cell size into account and is less suited to distinguish background staining compared to a cell-based approach. To our knowledge, the present study is the first study in LSCC or HNSCC that applies a cell-based, rather than pixel-based analysis to compare two hypoxia markers.

Digital analysis on a cellular level is relatively novel. While the reliability of scoring HIF-1α and PIMO expression has not been assessed in particular, other markers have already been validated. One study compared reproducibility for Ki-67 scoring (which is a nuclear DAB-staining like HIF-1α) and found a high inter-platform and inter-operator reproducibility for digital image analysis in general and in QuPath specifically.[39] Other studies have compared pathologist examination versus QuPath for CD8+ tumor-infiltrating lymphocytes (TILs) and PD-L1 and found a high concordance between the two.[40,41] Still, automated scoring of HIF-1α and PIMO staining as well as the hotspot detection methods should be validated against scoring by an experienced pathologist.

A limitation of cell detection analysis in QuPath is that it can detect but not interpret staining. Indeed we identified some areas where the cell detection was not perfect. Because of this it was still necessary to perform manual corrections and to remove areas of necrosis, scanning artifacts and areas of foldover from the automatically established annotations. To address this issue, QuPath extensions are rapidly becoming available to improve cell detection for instance using deep-learning methods.[42]

Conclusion

In summary, this is the first study to compare the immunohistochemical hypoxia markers HIF-1α and PIMO in LSCC using a digital, cell-based analysis. Our study shows that it is feasible to use open-source software to compute positive cell percentages and to investigate overlap of the two biomarkers in digitized sections in LSCC. However, these digital methods should be further validated against the current reference standard of visual scoring by a pathologist. In this relatively small study, we were unable to identify HIF-1α, or a combination of HIF-1α and PIMO as a marker to predict response to the additional effect of ARCON over AR alone. To our knowledge, this is the first study that uses automated digital imaging technology to show spatial correlations of HIF-1α and PIMO staining in HNSCC. We found a weak correlation between positive cell fractions for the two biomarkers, and a moderate correlation between the number of hypoxic regions for each biomarker. The moderate strength of this correlation in combination with the poor overlap of HIF-1α and PIMO suggests distinct differences between these two hypoxia biomarkers and also highlights the co-existence of different forms of hypoxia in a single tumor.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The original Phase III trial was supported by the Dutch Cancer Society (KWF) Research Fund No. CKTO-2000-09 and KUN-2008-4088 and METOXIA (Metastatic Tumors Facilitated by Hypoxic Tumor Micro-Environments; European Community Grant No. FP7-HEALTH-2007-B222741. No additional funding was received for the present study.

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Supplementary information S1.

Cell detection settings

HIF-1α staining	runPlugin('qupath.imagej.detect.cells.PositiveCellDetection', '{"detectionImageBrightfield": "Optical density sum", "requestedPixelSizeMicrons": 0.5, "backgroundRadiusMicrons": 30.0, "medianRadiusMicrons": 2.0, "sigmaMicrons": 2.5, "minAreaMicrons": 10.0, "maxAreaMicrons": 400.0, "threshold": 0.18, "maxBackground": 0.0, "watershedPostProcess": true, "excludeDAB": false, "cellExpansionMicrons": 7.0, "includeNuclei": true, "smoothBoundaries": true, "makeMeasurements": true, "thresholdCompartment": "Nucleus: DAB OD mean", "thresholdPositive1": 0.65, "thresholdPositive2": 0.4, "thresholdPositive3": 0.6, "singleThreshold": true}')
PIMO staining	runPlugin('qupath.imagej.detect.cells.PositiveCellDetection', '{"detectionImageBrightfield": "Hematoxylin OD", "requestedPixelSizeMicrons": 0.65, "backgroundRadiusMicrons": 35.0, "medianRadiusMicrons": 3.0, "sigmaMicrons": 2.0, "minAreaMicrons": 10.0, "maxAreaMicrons": 400.0, "threshold": 0.025, "maxBackground": 0.0, "watershedPostProcess": true, "excludeDAB": false, "cellExpansionMicrons": 5.0, "includeNuclei": true, "smoothBoundaries": true, "makeMeasurements": true, "thresholdCompartment": "Cytoplasm: DAB OD mean", "thresholdPositive1": 0.1, "thresholdPositive2": 0.4, "thresholdPositive3": 0.6, "singleThreshold": true}')

Hotspot detection settings

The script provided on https://gist.github.com/Svidro/6171d6d24a85539d3af5d417bc928d50#file-hotspot-detection-0-2-0m8-groovy requires setting the three parameters shown in the table below. Identical settings were used for each marker

Parameter	Definition	Settings
minCells	minimum number of cells in a hotspot	10
radiusMicrons	Distance between cells	25
pixelDensity	Changes with the other variables, requires testing	1

Chapter 3

The correlation of hypoxia, PD-L1 expression and T-cell influx and the effect on patient outcomes in oropharyngeal squamous cell carcinoma

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Manuscript in preparation

Abstract

Background: Hypoxia, immune-cell influx and immune-evasion are tumor microenvironment factors that drive treatment resistance in cancer. Recent evidence suggests that the immunotherapy target Programmed-Death-Ligand-1 (PD-L1) is a direct target of the Hypoxia-inducible-factor 1a (HIF-1a) transcription factor.

Methods: Immunohistochemical stainings were performed in 274 OPSCC patients (44 HPV-positive) for tumor expression of HIF-1a and PD-L1 and for CD3, CD8 and PD-1+ tumor-infiltrating lymphocytes (TILs). Correlation analysis and multivariate overall survival (OS) analysis were performed.

Results: There was a negative correlation between HIF-1a and CD3+/CD8+ TILs. Higher CD3+, CD8+ and PD-1+ TILs all were correlated with PD-L1 positivity. There was no correlation between HIF-1a and PD-L1 expression. In multivariate analysis, age, T- and N-classification, CD3+ TILs, HIF-1a and HPV-status were all independent predictors of OS.

Conclusions: Hypoxia and low TIL invasion were correlated and were both independent prognostic factors of worse OS. Hypoxia-targeted therapy and immunotherapy might be synergistic treatments.

Introduction

In the tumor microenvironment, there is constant interaction between a tumor and the host immune system.[1] The oropharynx is rich in lymphoid tissue, and there is a high influx of tumor infiltrating lymphocytes (TILs).[2] Several studies in oropharyngeal squamous cell carcinoma (OPSCC) show that high T-cell influx is related to better overall and disease-specific survival.[2–4] This may reflect a better capability of the host immune system to detect and eradicate tumor cells. Unfortunately, tumor cells interact with the immune system on several immune checkpoints and may inhibit the anti-tumor response. One of these checkpoints involves the programmed cell-death receptor-1 (PD-1) expressed on T-cells and its ligand PD-L1 (alternatively known as B7-H1 or CD274) on tumor cells. Interaction between PD-1 and PD-L1 leads to disengagement between T-cell and the tumor cell and also increases T-cell migration through the tissue, thus reducing the anti-tumor immune response.[5]

Recently, a direct link between tumor hypoxia and PD-L1 expression has been described.[6,7] Tumor hypoxia is a common characteristic of solid tumors and occurs when there is a mismatch between oxygen demand and supply.[8] Under chronic hypoxia, the Hypoxia-inducible factor 1-alpha (HIF-1a) is stabilized and together with its HIF-1 beta subunit forms the HIF-1 transcription factor, causing transcription of various proteins through hypoxia-responsive elements (HREs) in the DNA.[9] This transcriptional response increases cellular survival and prevents apoptosis under hypoxic circumstances.[8] For instance, targets of HIF-1a include proteins involved in pH regulation (carbonic anhydrase IX, CA-IX), glucose transport (glucose transporter 1, GLUT-1) for anaerobic metabolism and vessel formation (vascular endothelial growth factor, VEGF).[10] Previous studies have shown that overexpression of HIF-1a results in adverse prognosis in patients with OPSCC.[11,12]

PD-L1 has recently been described to be a target of HIF-1a. Barsoum *et al* described that PD-L1 protein expression was increased under hypoxia, and found two HREs in the PD-L1 gene.[6] Inhibition of HIF-1a using siRNAs directed against HIF-1a decreases PD-L1 expression.[6,7] Moreover, these studies found that hypoxia reduced tumor-cell killing by T-cells. Knockdown of either HIF-1a or PD-L1 using siRNA restored the cell-killing capabilities to normoxic levels. These results confirm interaction between tumor characteristics, such as hypoxia, and the host immune response *in vitro*.

In the present study we investigated invasion of CD3+, CD8+ and PD-1+ T-cells in combination with PD-L1 and HIF-1a expression in tissue of 274 OPSCC patients. The aim of this study was to gain insight on the correlation between these three factors and how they relate to overall survival in OPSCC patients. We hypothesized that high levels of TILs are beneficial to patient prognosis and that high levels of HIF-1a expression results in poor prognosis. Moreover, we expected that high levels of PD-L1 expression is a poor

prognosticator in OPSCC. In addition, a sub-study was performed to investigate the effect of hypoxia on T-cell activation.

Materials and Methods

Patient cohort

Patients were included who participated in previously described, retrospective cohorts.[13,14] These were 274 patients diagnosed with a first primary OPSCC between 1997 and 2011 at the departments of Otorhinolaryngology – Head and Neck Surgery or Oral and Maxillofacial Surgery in the University Medical Center Utrecht. All patients who had sufficient tissue available for creation of a tissue microarray (TMA) were included. Patients were excluded if they had a previous head and neck malignancy, or if treatment was not with curative intent (i.e. palliative treatment). After discussing the patient in the multidisciplinary head and neck oncology team meeting, the definitive treatment strategy was decided by the patient and their treating physicians. As leftover material was used for this study and all data were handled anonymously, obtaining ethical approval or informed consent was not required according to Dutch law and 'Best practice' guidelines on research ethics.[15] All data are reported according to the REMARK recommendations for tumor marker prognostic studies.[16] Data were handled according to GDPR.

Immunohistochemical analysis

All tissues were included in a TMA. In brief, tissue was obtained from formalin fixed and paraffin embedded (FFPE) tissue blocks that had been stored in the archives at room temperature. Sections of the FFPE blocks were stained with hematoxylin and eosin (H&E) and assessed by a dedicated head and neck pathologist (SMW) to mark representative tumor regions. Three 0.6 mm tumor cores per patient were introduced in a recipient TMA block. From this TMA, 4 μm thick sections were cut and stained. The PD-1, PD-L1, CD3 and CD8 stainings were performed using a Ventana autostainer (Ventana Medical Systems, Inc, Roche, USA). The HIF-1a stainings were performed manually using the Novolink kit (Leica, Eindhoven, the Netherlands) for development, as previously described.[14] After development, the tissues were counterstained with hematoxylin. The antibodies that were used were F7.2.38 for CD3, C8/144B for CD8, MIB-1 for Ki-67 (Agilent Dako, Santa Clara, USA), 54/HIF-1α for HIF-1a (BD Biosciences, New Jersey, USA), NAT105 for PD-1 (Abcam, Cambridge, UK) and SP-263 for PD-L1 (Roche Ventana, Arizona, USA). The exact staining procedures and reagents are described in **Supplementary Table S1**.

After staining, protein expression and TIL numbers were scored by an experienced head and neck pathologist and an ENT-resident/medical researcher in consensus. For PD-L1 and HIF-1a, the percentage of positive tumor cells was semi-quantitatively assessed. If cores were

folded or contained less than 25% tumor tissue, they were excluded from analysis. Cores were regarded missing if more than 5% was lost. For PD-L1 and HIF-1a, only membranous and nuclear staining respectively were considered biologically relevant. For PD-1, CD8 and CD3-positive TILs the absolute numbers of positive cells were counted. Because the cores were consistent in size, no further normalization for the area was necessary. The average score of the evaluable cores was calculated and used for further analysis. For HIF-1a, positivity was set at 15% or more expressing cells in concordance with our previous study.[14] For CD3+ and CD8+ TILs, 150 and 100 or more cells, respectively, were considered positive based on previous studies.[17] As, to our knowledge, expression of PD-1 has not been investigated in a similar way, we have established cut-off points for positivity using optimum stratification.[18] The optimal cut-off point with the strongest relation to overall survival and was found to be 70 cells per core. The cut-off value for PD-L1 positivity was set at 1% or more positive cells based on the literature.[1,19] Tumor HPV-status had been determined for clinical purposes apart from the study using IHC for p16, followed by a HPV-PCR when positive.[20]

T-cell activation sub-study

To further characterize the effect of hypoxia on TILs a sub-study was performed in ten patients. These were five patients with HIF-1a positive and five with HIF-1a negative tumors chosen at random. Besides hypoxic status, the only criterion for inclusion in this sub-study was a number of T-cells between 100 and 150 per TMA core so that a sufficient number of T-cells was available for analysis. An immunofluorescence triple staining was performed for HIF-1a (hypoxia marker), CD3 (T-cell marker) and CD-69 as a T-cell activation marker.

Endogenous peroxidase activity was blocked with a 1.5% H2O2 solution for 15 minutes, followed by antigen retrieval in a pH 9 EDTA buffer. Non-specific antibody binding was prevented using a 1% BSA solution for 30 minutes. The sections were incubated for 1 hour at room temperature with rat anti-CD3 (Abcam, ab11089) and rabbit anti-CD69 (Atlas Antibodies, HPA050525) antibodies diluted at 1:200 in the BSA solution. The sections were washed with PBS and incubated with the secondary antibodies Alexa Fluor 488-anti-rat (Invitrogen, a21208) and Alexa Fluor 647 anti-rabbit (Invitrogen, a21245) at 1:200 for one hour at room temperature before being washed again. Additional blocking was carried out with protein block from the Novolink kit (Leica Biosystems, Novolink Polymer Detection Systems) for 30 minutes before washing and incubating with Mouse anti-HIF1a (BD Biosciences, 610958) at 1:50 overnight at 4°C. The post-primary block and polymer were both applied for 30 minutes. The Opal 570 reagent (Akoya Biosciences, FP1488001KT) was applied diluted 1:200 in 1x plus amplification diluent (Akoya Biosciences, FP1498) for 10 minutes at room temperature. The tissue sections were then washed and stained with DAPI for 15 minutes at room temperature. The sections were washed a final time, covered with

ProLong Diamond Antifade Mountant (Thermo Fisher, P36965) and coverslipped. The resulting stains were imaged by a Zeiss LSM 700 confocal microscope at 20x magnification.

The number of activated T-cells (co-expression of membranous CD3 and CD69 staining) was assessed semi-quantitatively by two researchers in consensus as low (<5% activated T-cells), intermediate (10-50% activated T-cells) and high (> 50% activated T-cells).

Statistical analysis

Missing data were handled by creating 10 multiple imputation datasets. The exact imputation strategy is shown in **Supplementary Data S2**. For descriptive analyses, the original, non-imputed, data are reported. For all statistical analyses, including correlations and survival analyses, the pooled imputed data were used. Normality was investigated by visual analysis using Q-Q plots, as well as using a Kolmogorov-Smirnov test. TIL-counts were not normally distributed, therefore a log-transformation was applied.

T-tests were used to compare normally distributed variables across groups. For nonparametric variables, an independent samples median test was performed. Categorical variables were compared using chi-squared analyses. For correlation analyses, Pearson correlation was used when comparing two continuous variables. When correlating a continuous variable with a dichotomous variable, logistic regression was used. For correlations between PD1+ TIL count and clinical variables, univariate and multivariate regression analysis were performed.

For overall survival (OS) analysis we measured from the date of first positive biopsy, until the date of death (event) or the date where the patient was last confirmed alive (censor). All available follow-up data were used; there was no loss to follow-up. For the locoregional control (LRC) analyses, we measured from the date of first positive biopsy, until the date of locoregional disease (event) or the last date where the patient had been seen and physically examined in our outpatient clinic (censor). Cox-regression was used for univariate and multivariate survival analyses. All clinical and pathological variables described in the baseline table were introduced into the univariate analysis. Variables were introduced in the multivariate analysis if they had a significant relation to OS in univariate analysis, or if the variables were likely to have relation with survival according to expert opinion of the authors. Multivariate Cox-regression was performed using backward selection with a p-value of 0.157 as exclusion limit, also known as Akaike's Information Criterion, AIC.[21] Kaplan-Meier survival curves were constructed to depict survival.

Results

Patient characteristics

Of the 274 OPSCC patients, most (69.3%) were male with a mean age of 59.3 years (**Table 1**). The greater majority of patients had a history of smoking (90.1%) and/or alcohol use (88.9%). Most patients presented with locally advanced tumors (T3-4, 57.7%) and around two-third of patients presented with lymph node metastasis at baseline. HPV-status was positive in 16.8% of patients and in 60.9% of patients the tumor was localized in the tonsil or base of tongue. The greater majority (67.5%) was treated with radiation therapy with or without concurrent cisplatin-based chemotherapy.

Immunohistochemistry

Due to loss of TMA cores in the staining process, HIF-1a staining was available for 260 patients (94.9%), PD-1 staining for 259 patients (94.5%), PD-L1 staining for 212 (77.4%), CD3 staining for 255 patients (93.1%) and CD8 staining for 246 patients (89.8%). A representative image of all stains is shown in **Figure 1**.

HIF-1a staining was positive in 143 patients (55%). Median expression was 23% positive cells (IQR 4 – 46% positive cells). PD-L1 staining was positive in 120 patients (56.8%). Median expression was 2% positive cells (IQR 0 – 15% positive cells). A median of 23 (IQR 8 – 46) PD-1+ TILs was present per TMA core. High numbers of PD1+ TILs (>70 PD-1 positive TILs per TMA cores) were present in 32 patients (12.4%). There was a median of 68 (IQR 37 – 132) CD3+ TILs present per TMA core. High numbers of CD3+ TILs (>150 cells per TMA core) occurred in 52 patients (20.4%). There was a median of 48 (IQR 25 – 94) CD8+ TILs present. High numbers of CD8+ TILs (>100 cells per TMA core) occurred in 52 patients (21.1%).

Correlations between clinical characteristics, PD1+ TILs and PD-L1

We first investigated the correlation between PD-1+ TILs and PD-L1 expression with clinical characteristics. These are shown in **Table 2**. Interestingly, there was a significant correlation between a patients smoking history and tumor invasion of PD1+ TILs. The mean number of PD1+ TILs was also higher in patients with nodal metastases and in patients with HPV+ tumors. Multivariate analysis showed that these correlations were mediated by HPV-status. In a multivariate regression analysis of PD1+ TILs with HPV and smoking status, smoking did not have a significant correlation with the number of PD1+ TILs, while HPV-status did. The same was true for clinical N-stage: In multivariate linear regression for PD1+ TILs with HPV and N-stage, N-stage did not have a significant correlation while HPV-status

Characteristic	Patients *n = 274*
Age (*years*)	
Mean (SD)	59.3 (9.1)
Sex	
Male	190 (69.3)
Female	84 (30.7)
Smoking	
Never ever	27 (9.9)
Quit / active smoker	245 (90.1)
Alcohol use	
Never ever	30 (11.1)
Current or previous use	240 (88.9)
Clinical T-classification	
T_1	36 (13.2)
T_2	78 (28.6)
T_3	56 (20.5)
T_{4a}/T_{4b}	103 (37.7)
Clinical N-classification	
N_0	96 (35.3)
N_1	38 (14.0)
$N_{2a/b/c}$	125 (46.0)
N_3	13 (4.8)
HPV-status	
Positive	44 (16.8)
Negative	218 (83.2)
Localization	
Tonsil/base of tongue	167 (60.9)
Other	107 (39.1)
Differentiation	
Well	4 (2.0)
Moderate	146 (71.6)
Poorly	52 (25.5)
Undifferentiated	2 (1.0)
Treatment	
Surgery only	15 (5.5)
Surgery + PORT	69 (25.2)
Surgery + POCRT	5 (1.8)
Primary RT	74 (27.0)
Primary CRT	111 (40.5)
HIF-1a	
Positive (≥ 15%)	143 (55.0)
Negative (< 15%)	117 (45.0)
Follow-up	
All patients median (IQR)	35.0 (16.0 – 67.0)
Surviving patients median (IQR)	63.0 (39.8 – 80.0)
Deceased at end of follow-up	152 (55.5)

Table 1 *(previous page)* | Baseline patient characteristics. Baseline patient data are shown before missing data imputation was applied. Data are provided as n (%), unless stated otherwise. Where total numbers do not reach 274, this was because of missing data. Please find supplementary data SD1 for missing data analysis.

did. For PD-L1 the only clinical variable that had a significant correlation was HPV: patients with HPV-positive tumors were more likely to have PD-L1 positive tumors.

Correlations between immunohistochemical markers

There was a weak but significant correlation between PD-L1 positivity and the number of PD1, CD3+ and CD8+ TILs (**Table 3**). The correlation between PD-L1 positivity and the number of CD8+ TILs was stronger but still a weak correlation. As expected, there was a significant moderate to strong correlation between CD3+, CD8+ and PD1+ TILs. HIF-1a positivity had a significant negative correlation with the number of CD3+ and CD8+ TILs. There was no significant correlation between HIF-1a positivity and either PD-L1 positivity or the number of PD1+ cells.

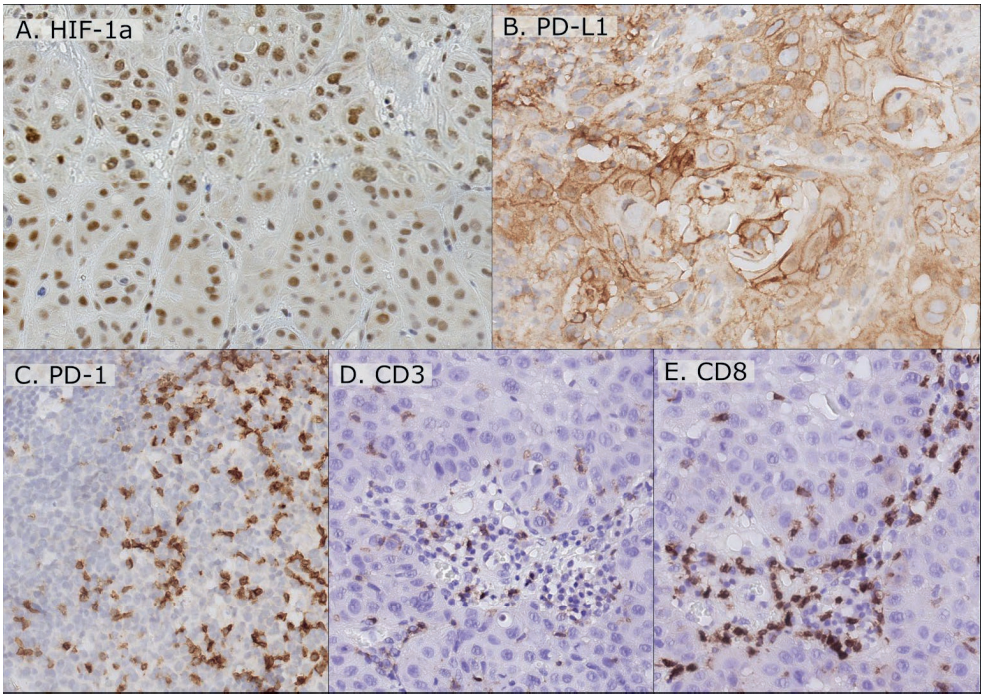

Figure 1 | Staining examples of HIF-1a, CD3, CD8, PD-1 and PD-L1

Variable	PD-1+ TIL count Median (IQR)	p	% PD-L1 positive	p
Sex*		NS		NS
Male	25.0 (8.3 – 46.4)		66.3	
Female	25.4 (10.0 – 51.9)		65.0	
Age†		NS		NS
Smoking		0.003		NS
Never	43.4 (26.3 – 76.3)		84.1	
Quit/Active	21.7 (8.3 – 45.0)		63.9	
Alcohol use		NS		NS
Never	33.3 (11.7 – 63.3)		73.2	
Quit/Active	22.5 (8.3 – 45.7)		64.9	
Clinical T-stage		NS		NS
cT1-2	25.0 (7.5 – 50.0)		63.9	
cT3-$4_{a/b}$	25.0(10.0 – 47.0)		67.3	
Clinical N-stage		0.035		NS
cN0	19.2 (6.9 – 37.9)		63.0	
cN+	28.3 (10.0 – 57.7)		67.7	
Differentiation grade		NS		NS
Moderate/Well	20.7 (8.3 – 45.0)		64.6	
Undifferentiated/Poor	32.1 (15.2 – 60.0)		68.1	
HPV-status		<0.001		0.017
Negative	20.0 (8.3 – 42.0)		62.6	
Positive	51.3 (29.6 – 83.4)		82.6	

Table 2 | Correlations of PD-1 and PD-L1 to clinical variables. For PD-L1, the percentage of PD-L1 positive patients (PD-L1 expression in 1% of tumor cells or more) within a group is shown. Significant differences are shown in bold. NS: not significant. * The illustrated median numbers of PD1 positive TILs per category are shown from the first imputed database. † For PD-1 count, Pearson correlation was used, for PD-L1 positivity, logistic regression was used.

Protein		PD-L1 positivity	PD1+ TILs	CD3+ TILs	CD8+ TILs
PD1+ TILs	*r*	0.150			
	p	.014			
CD3+ TILs	*r*	0.175	0.564		
	p	.005	<0.001		
CD8+ TILs	*r*	0.278	0.607	0.801	
	p	<0.001	<0.001	<0.001	
HIF-1a positivity	*r*	-0.007	-0.044	-0.157	-0.154
	p	0.909	0.483	0.015	0.017

Table 3 | Correlations between TILs, PD-L1 and HIF expression. Abbreviations: *r* - correlation-coefficient, TILs – tumor infiltrating lymphocytes.

Survival analyses

Survival analyses are shown in **Table 4**. The characteristics age, smoking history, clinical T- and N- stage, HPV status, high numbers of PD1+, CD3+ and CD8+ TILs and HIF-1a positivity were significantly related to OS in univariate analysis (**Figure 2**). HIF-1a and PD-L1 co-expression was not significantly related to OS or LRC. In multivariate analyses, only age, T- and N-stage, HPV-status, HIF-1a positivity, high numbers of CD3+ TILs and treatment strategy were significantly related to OS. PD-L1 positivity was not significantly correlated to OS. In the locoregional control analysis, only the number of CD8+ TILs was associated with LRC. This is shown in Table S1. Because only one variable tested significant, no multivariate analysis could be performed.

Variable	Univariate analysis			Multivariate analysis		
	HR	95% CI	p	HR	95% CI	p
Age	1.02	1.00 – 1.04	0.021	1.03	1.01 – 1.05	0.004
Sex						
male vs female	0.27	0.57 – 1.17	NS	-	-	-
Smoking						
active/quit vs. never	2.27	1.11 – 4.60	0.025	NA	NA	NA
Alcohol						
active/quit vs. never	1.40	0.80 – 2.47	NS	-	-	-
Clinical T-classification						
T3-$4_{a/b}$ vs. T1-2	2.07	1.46 – 2.92	<0.001	1.72	1.12 – 2.64	0.014
Clinical N-classification						
N+ vs. N0	1.65	1.16 – 2.35	0.006	2.32	1.56 – 3.48	<0.001
Differentiation grade						
III-IV vs. I-II	0.68	0.42 – 1.09	NS	-	-	-
HPV						
positive vs. negative	0.29	0.15 – 0.56	<0.001	0.33	0.16 – 0.67	0.002
Subsite						
Tonsil/base of tongue vs other	1.00	0.72 – 1.38	NS	-	-	-
PD-1+ TILs						
high vs. low	0.54	0.30 – 0.96	0.035	NA	NA	NA
PD-L1						
positive vs. negative	0.96	0.68 – 1.36	NS	NA	NA	NA
CD3+ TILs						
high vs. low	0.41	0.25 – 0.69	<0.001	0.48	0.28 – 0.82	0.007
CD8+ TILs						
high vs. low	0.41	0.24 – 0.68	0.001	NA	NA	NA
HIF-1a						
positive vs. negative	1.56	1.11 – 2.19	0.010	1.52	1.08 – 2.15	0.017
Treatment strategy						
Surgery vs CRT	0.55	0.25 – 1.19	NS	0.92	0.39 – 2.16	NS
Surgery + PORT vs CRT	0.55	0.36 – 0.82	0.004	0.49	0.31 – 0.78	0.003
Surgery + POCRT vs CRT	0.90	0.28 – 2.85	NS	0.72	0.23 – 2.33	NS
Primary RT vs CRT	0.59	0.39 – 0.89	0.011	0.95	0.58 – 1.57	NS

Table 4 *(previous page)* | Overall survival analyses. All variables that were significant in univariate analysis were included in the multivariate analysis. PD-L1 was included based on clinical relevance. For treatment strategy, the largest category was chosen as the reference category. Abbreviations: NS – not significant; NA – removed by backwards regression

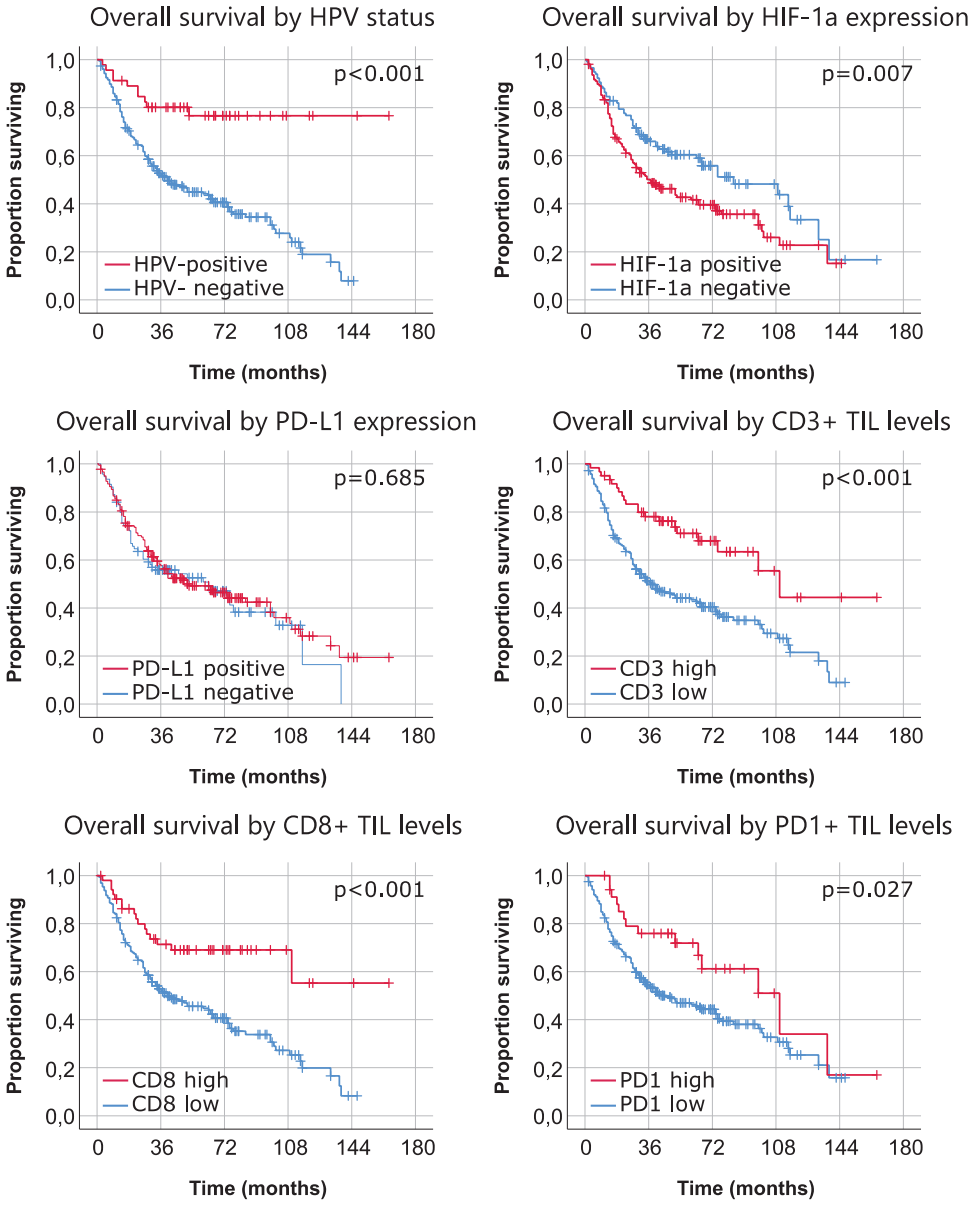

Figure 2 | Kaplan-Meier survival curves. Overall survival curves are shown for HPV-status, HIF-1a expression, PD-L1 expression, and CD3+, CD8+ and PD1+ TIL levels

T-cell activation sub-study

To identify whether hypoxia did not only decrease the number of TILs, but also influenced T-cell activation, we performed a sub-study in ten patients. We found that the number of activated T-cells did not differ according to the hypoxic status. The number of activated T-cells was low (n=2 in each group), intermediate (n=1 in each group) and high (n=2 in each group). An example of a high number of activated T-cells is shown in **Figure 3**.

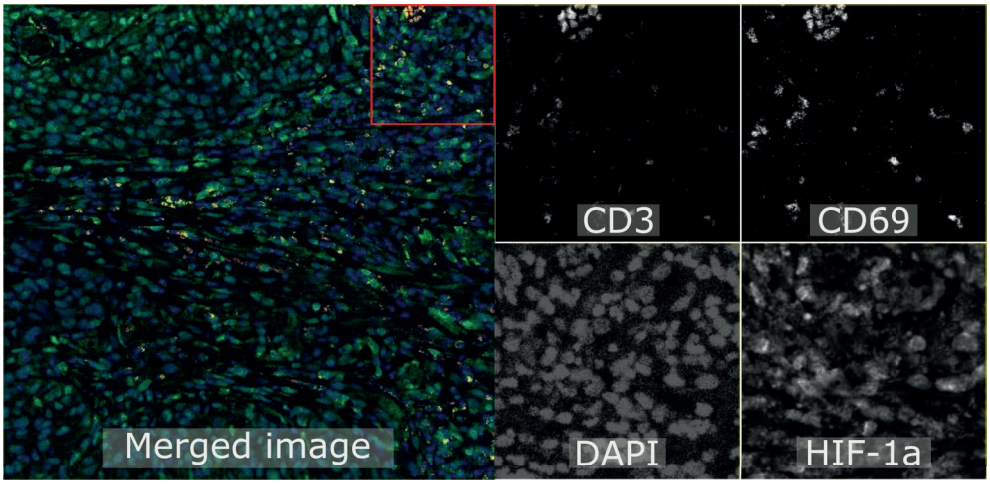

Figure 3 | Immunofluorescence triple staining of HIF-1a (hypoxia marker), CD3 (T-cell marker) and CD-69 (T-cell activation marker). Representative tissue section area showing HIF-1a staining in green, CD3 staining in red and CD69 staining in yellow. The DAPI staining is shown in blue (20x magnification). The individual grayscale channels of the area in the red square are shown in more detail to the right.

Discussion

In this study we investigated the correlation between biomarkers for hypoxia and immune infiltration in a cohort of patients with OPSCC. In this well described patient cohort, we found no correlation between PD-L1 and HIF-1a overexpression. Expression of PD-L1 and co-expression of HIF-1a and PD-L1 were not related to overall survival. We did find that HIF-1a was correlated to lower numbers of TILs and that both these factors were independent prognostic factors of worse OS.

The awareness increases that in solid tumors, not only the size and extent of metastasis affect therapy sensitivity. Rather, solid tumors encompass a microenvironment, in which not only the molecular characteristics of the neoplastic cells, but also the presence of immune cells and characteristics such as hypoxia play a role.[8,22] We have previously described that

high HIF-1a expression, a marker for tumor hypoxia, is related to worse overall survival.[14] In the present study we investigated how HIF-1a expression correlates to immunological factors within the tumor microenvironment.

Hypoxia-responsive elements (HREs) in the genome are targets for HIF-transcription factors.[23] *In vitro*, Barsoum and colleagues have demonstrated that HIF-1a and PD-L1 are both upregulated under hypoxia.[6] They describe that PD-L1 is a downstream target of HIF, by demonstrating the presence of a HRE in the promotor region of the PD-L1 gene. Upregulation of PD-L1 under hypoxia through HIF-1a was also observed by Noman and colleagues.[7] There is limited clinical evidence on this correlation in HNSCC patients.

In our large cohort of OPSCC patients, we found no correlation between HIF-1a and PD-L1 protein expression. This is in contrast with findings by Chen and colleagues in oral squamous cell carcinoma who found a high correlation between HIF-1a and PD-L1 positivity in primary tumors and even stronger in metastases.[24] Another study in 63 HNSCC patients (mostly laryngeal carcinoma) describes a strong predictive potential of the combination of HIF-1a positive and PD-L1 positive status for OS.[25] The correlation between HIF-1a and PD-L1 expression was not described in this study. It is unclear why we were unable to find a correlation between HIF-1a and PD-L1 in the present study. This analysis should be repeated in other OPSCC patient cohorts.

We did not find any prognostic value of PD-L1 for LRC or OS, which is in line with a recent meta-analysis on this subject.[26] Moreover, PD-L1 expression was higher in patients with HPV+ OPSCC, which is also in line with the literature.[26] Presence of PD-1+ TILs, as well as the number of CD3+ and CD8+ TILs were prognostic for OS, but in multivariate analysis, CD3+ TILs was the strongest of these three predictors and the only to retain significance. It should be noted that as no cut-off points for the number of PD-1+ TILs has been established in the literature we have used the optimal stratification cutoff point.[18] This cutoff point should be validated in future studies.

To the authors' knowledge, this is the first study to investigate the correlation between HIF-1a and TILs in HNSCC patients. We found an inverse correlation between tumor HIF-1a expression and CD3+ TIL invasion in OPSCC patients. This finding is in line with several previous *in vitro* studies on CD3+ lymphocyte function and proliferation under hypoxia as reviewed by Kumar and colleagues.[27] Hypoxia and HIF-1a stabilization in both tumor cells and T-cells have been shown to impair the general and tumor-directed immune response. Proliferation and function of T-lymphocytes has been shown to decrease under decreased oxygen levels *in vitro*.[28] Moreover, HIF-1a deficient T-cell lines have been shown to significantly secrete more pro-inflammatory cytokines in both normoxic and hypoxic conditions.[29] In tumor cells, hypoxic HIF-1a stabilization reduces cytotoxic T-cell mediated lysis, which was restored when cells were transfected with siRNAs directed against HIF-1a.[7,30]

In a small exploratory sub-study on the effect of HIF-1a expression on T-cell activation we could not verify the aforementioned preclinical studies that describe that T-cell activation is lower in hypoxic tumors. However, our sub-study may well have been underpowered to significantly detect smaller effects.

There is strong evidence of interactions between tumor hypoxia, HIF-1a and the anti-tumor immune response. Therefore, a combination of hypoxia or HIF-1a targeted therapy and immunotherapy might well have a synergistic effect by increasing TIL-invasion and function. While hypoxic radiosensitizing drugs such as Nimorazole have shown clinical effect on survival, they are less likely to influence the tumor's hypoxic state and the anti-tumor immune response through hypoxia. Direct inhibition of HIF-1a has recently been shown to increase the antitumor response by increasing the number of CD8+ and NK-cells in the microenvironment.[31] This study also showed improved effectivity of cancer immunotherapy in patients treated with a pharmacological agent that prevents HIF-1α and HIF-1β dimerization.[31] Selective inhibition of HIF-1a using BAY 87-2243 has been shown to decrease downstream target transcription *in vitro* and to increase radiosensitivity *in vivo* in mouse models.[32,33] The effect of HIF-1a inhibition on PD-L1 expression, T-cell function and their subsequent clinical effects should be further investigated.

Several aspects of this study should be further discussed. First, all biomarkers were analyzed using a TMA and were scored by the same (blinded) observer. It has been shown that TMAs provide a representative sample of a whole tumor in biomarker expression studies.[34,35] Specifically for HIF-1a, a strong correlation has been described between TMA and whole biopsy assessment and false-positivity is rare.[36] In the case of TILs, their density may be heterogeneous throughout a tumor. However, the correlation between the number of TILs in a tumor's center and periphery has a strong correlation.[37] In addition discerning tumors with high from low numbers of TILs (i.e. ordinal categorization) using a TMA has been shown to be reliable.[38] The type of antibody used to analyze PD-L1 varies in clinical practice. The most often used assays include Sp263 and 22C3. For this study we used the Sp263 assay that is also used clinically using a Ventana autostainer. This specific assay partakes in an inter-laboratory audit in the Netherlands.

Conclusion

In conclusion, we provide clinical evidence that expression of HIF-1a in tumor cells is inversely correlated to T-cell influx and that these factors are independently associated with OS in OPSCC patients. In addition, while there is preclinical and clinical evidence in HNSCC that PD-L1 is a downstream target of HIF-1a we were unable to verify this finding in a large cohort of OPSCC patients. Still, our findings illustrate that factors within the tumor microenvironment are related and that therapy directed against one factor may be also

beneficial to other factors. We propose that hypoxia or HIF-1a-targeted therapy and immunotherapy might be synergistic treatments.

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Supplementary table S1. Staining procedures, reagents and antibodies

Immunohistochemistry							
Antigen	Antigen retrieval	Brand	Clone	Catalog ID	Dilution	Duration	Temperature
CD3	Ultra-CC1* (Ventana)	Dako	F7.2.38	A0452	1:400	32 min	36°C
CD8	Ultra-CC1* (Ventana)	Dako	C8/144B	20078591	1:100	32 min	36°C
HIF-1a	EDTA	BD Biosciences	54/HIF-1α	610959	1:50	Overnight	4°C
Ki-67	Ultra-CC1* (Ventana)	Dako	MIB-1	M7240	1:100	32 min	36°C
PD-1	Ultra-CC1* (Ventana)	Abcam	NAT105	B214288	1:100	32 min	36°C
PD-L1	Ultra-CC1* (Ventana)	Ventana	SP-263	E09452	None	32 min	36°C

* CC1 is an EDTA-based buffer solution

Supplementary data S2: missing data handling

Imputation was performed using multiple imputation that included 10 imputed datasets. This was performed in SPSS version 25.0. For quantitative variables the minimum and maximum values observed in the dataset were used as limits for the imputation.

Variable	Missing n (%)	Role in imputation	Min/max value
Age	0 (0)	P	35 – 83
Sex	0 (0)	P	-
Subsite	0 (0)	P	-
Smoking	2 (0.7)	P / I	-
Alcohol use	4 (1.5)	P / I	-
T-stage	1 (0.4)	P / I	-
N-stage	0 (0)	P	-
Differentiation grade	70 (25.5)	P / I	-
HPV-status	12 (4.4)	P / I	-
CD3 positive cells	19 (6.9)	P / I	6 – 580
CD8 positive cells	28 (10.2)	P / I	5 – 390
HIF-1a expression	14 (5.1)	P / I	0 – 95
PD1 positive cells	15 (5.5)	P / I	0 – 231
PD-L1 expression	62 (22.6)	P / I	0 – 98
Survival	0 (0)	P	-
Treatment modality	0 (0)	P	-

P: variable was used as a predictor, I: the variable was imputed

Imputation script

```
MULTIPLE IMPUTATION Age Sex Site smoking_dich alcohol_dich ct_dich cn_dich
Path_Differentiation HPV_HR CD3_mean CD8_mean
    HIF_mean PD1_mean PDL1_SP263_mean OS_Outcome TX_BSL
  /IMPUTE METHOD=AUTO NIMPUTATIONS=10 MAXPCTMISSING=NONE  MAXCASEDRAWS=50
MAXPARAMDRAWS=5
  /CONSTRAINTS Age( ROLE=IND)
  /CONSTRAINTS Sex( ROLE=IND)
  /CONSTRAINTS Site( ROLE=IND)
  /CONSTRAINTS cn_dich ( ROLE=IND)
  /CONSTRAINTS CD3_mean( MIN=6.0 MAX=580.0 RND=1.0)
  /CONSTRAINTS CD8_mean( MIN=5.0 MAX=390.0 RND=1.0)
  /CONSTRAINTS HIF_mean( MIN=0.0 MAX=95.0 RND=1.0)
  /CONSTRAINTS PD1_mean( MIN=0.0 MAX=231.0 RND=1.0)
  /CONSTRAINTS PDL1_SP263_mean( MIN=0.0 MAX=98.0 RND=1.0)
  /CONSTRAINTS OS_Outcome( ROLE=IND)
  /CONSTRAINTS TX_BSL( ROLE=IND)
  /MISSINGSUMMARIES NONE
  /IMPUTATIONSUMMARIES MODELS
  /OUTFILE  IMPUTATIONS='X:\AAD.  Promotie\Hypoxia\Ch  5.  PD-1,  PD-
L1\Data\PD1_PDL1 OPSCC '+ 'study_imputed260419.sav' .
```

Supplementary table S3. Locoregional control analysis

	Univariate analysis		
Variable	HR	95% CI	p
Age	0.99	0.96 – 1.02	NS
Sex			
male vs female	0.95	0.54 – 1.69	NS
Smoking			
active/quit vs. never	1.64	0.60 – 4.54	NS
Alcohol			
active/quit vs. never	1.76	0.64 – 4.89	NS
Clinical T-stage			
T3-$4_{a/b}$ vs. T1-2	1.13	0.66 – 1.93	NS
Clinical N-stage			
N+ vs. N0	1.19	0.68 – 2.09	NS
Differentiation grade			
III-IV vs. I-II	0.74	0.41 – 1.35	NS
HPV			
positive vs. negative	0.41	0.16 – 1.04	NS
PD-1+ TILs			
high vs. low	0.83	0.37 – 1.86	NS
PD-L1			
positive vs. negative	0.79	0.45 – 1.37	NS
CD3+ TILs			
high vs. low	0.64	0.29 – 1.37	NS
CD8+ TILs			
high vs. low	0.39	0.15 – 0.98	0.046
HIF-1a			
positive vs. negative	1.49	0.86 – 2.62	NS
Treatment strategy			
Surgery vs CRT	0.72	0.22 – 2.39	NS
Surgery + PORT vs CRT	0.54	0.27 – 1.10	NS
Surgery + POCRT vs CRT	1.38	0.33 – 5.80	NS
Primary RT vs CRT	0.60	0.31 – 1.19	NS

Chapter 4

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 (OPSCC).

Materials and methods: 20 patients with 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 1-alpha (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.

Highlights

- In OPSCC, high tumor proliferation rate correlates to lower ADCs in DW-MRI.
- In OPSCC, high CD3-positive lymphocyte influx correlates to lower ADCs.
- A correlation between tumor hypoxia and whole tumor ADC could not be identified.
- Better knowledge of the biological and microanatomical background of ADC should lead to better understanding of the role of DWI 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 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 1-alpha (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 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.

Variable	n (%)
Sex	
Male	11 (55)
Female	9 (45)
Age (mean, SD)	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 (mean, SD)	124 (78)
% HIF-1a expression (mean, SD)	32 (27)
% Ki-67 expression* (mean, SD)	32 (19)
ADC ($x10^{-3}$; mean, SD)	1.53 (0.31)
Total *n* (%)	20 (100)

Table 1 | Baseline patient and tumor characteristics. Variables are shown as *n* (%), unless stated otherwise.
* Ki-67 expression could not be determined for 1 patient.

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 **Figure 1**. The mean whole tumor ADC was 1.53×10^{-3} mm^2/s (range $1.18–2.28 \times 10^{-3}$ mm^2/s, SD = 0.31×10^{-3}). An example of a DWI-scan is shown in **Figure 2**.

The linear regression analyses between histological characteristics and the ADC values is shown in **Figure 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 −0.033, p = .024). There was a significant correlation between the ADC and CD3 positive cell count (*r* = −0.568, 95%

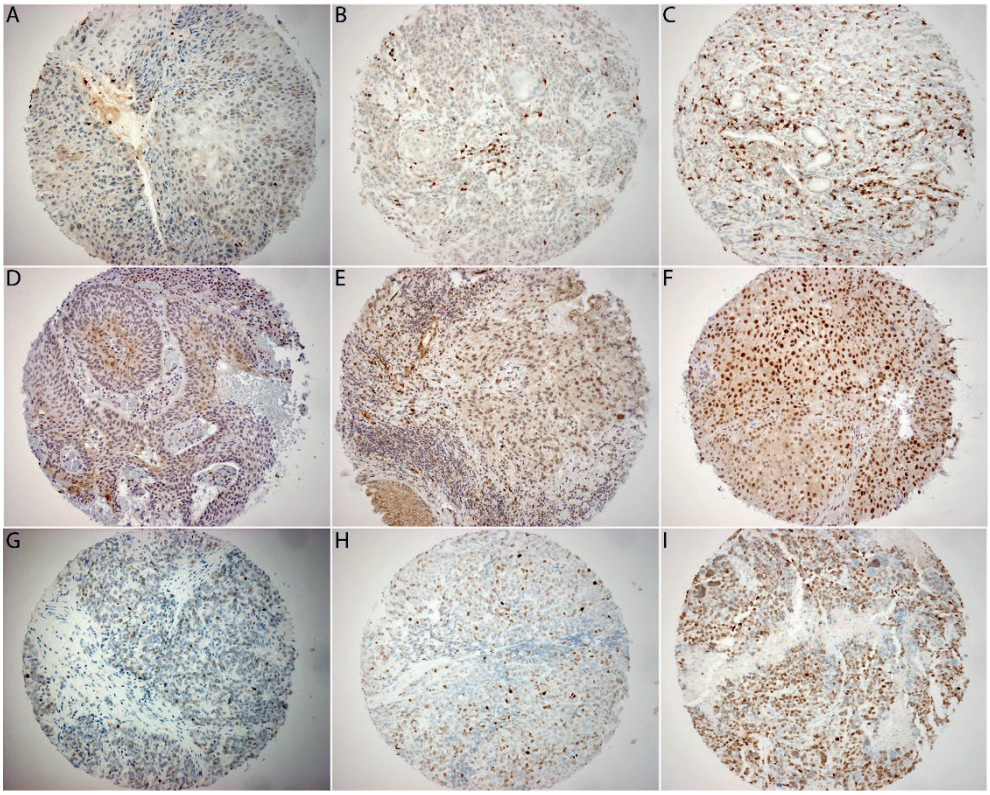

Figure 1 | Immunohistochemical staining. Staining examples of CD3 (A-C), HIF-1a (D-F) and Ki-67 staining (G-I). In A, B and C, there are 5, 70 and 280 CD3+ positive cells, respectively. D shows 5% HIF-1a positive tumor cells, E and F show 20 and 80% of HIF-1a positive tumor cells. G, H and I show 10,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

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).*

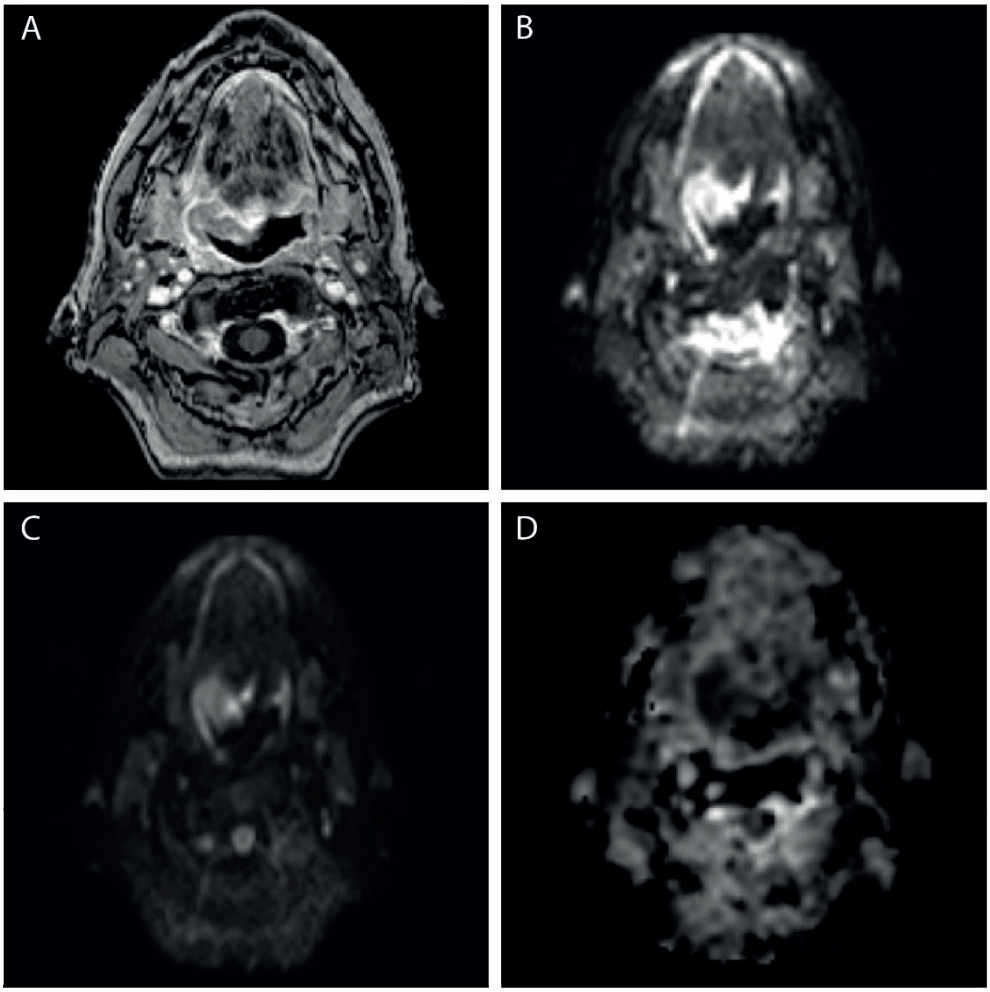

Figure 2 | Diffusion-weighted imaging. Axial MR images in a 61 year-old male with an oropharyngeal carcinoma centered in the right base tongue. Axial post-contrast T1-weighted MR image with fat suppression (A), axial DW image b = 0 s/mm^2 (B) and b = 800 s/mm^2 (C). The corresponding axial ADC map is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

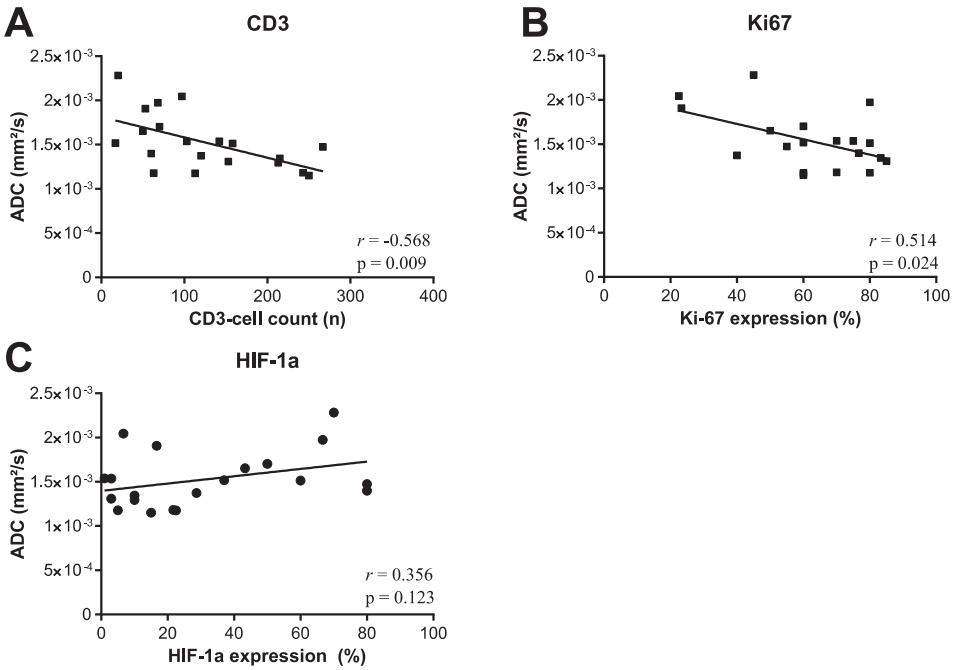

Figure 3 | Correlations between histological markers and DW-MRI. A significant inverse correlation was observed between CD3-cell count and ADC (A). Ki67 significantly inversely correlated to ADC (B). No correlation was observed between HIF-1a expression and ADC (C)

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, little is known about the biophysical background of DWI and an explanation to why ADC is able to predict outcome remains unclear.[3,4] 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 water molecules within the tumor stroma, larger cell-to-stroma ratios due to proliferation may lead to lower ADC. [5]

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 microanatomical 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.

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Chapter 5

Clinical implications of hypoxia biomarker expression in head and neck squamous cell carcinoma: a systematic review

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Abstract

Awareness increases that the tumor biology influences treatment outcome and prognosis in cancer. Tumor hypoxia is thought to decrease sensitivity to radiotherapy and some forms of chemotherapy. Presence of hypoxia may be assessed by investigating expression of endogenous markers of hypoxia (EMH) using immunohistochemistry (IHC). In this systematic review we investigated the effect of EMH expression on local control and survival according to treatment modality in head and neck cancer (head and neck squamous cell carcinoma [HNSCC]). A search was performed in MEDLINE and EMBASE. Studies were eligible for inclusion that described EMH expression in relation to outcome in HNSCC patients. Quality was assessed using the Quality in Prognosis Studies (QUIPS) tool. Hazard ratios for locoregional control and survival were extracted. Forty studies of adequate quality were included. HIF-1a, HIF-2a, CA-IX, GLUT-1, and OPN were identified as the best described EMHs. With exception of HIF-2a, all EMHs were significantly related to adverse outcome in multiple studies, especially in studies where patients underwent single-modality treatment. Positive expression was often correlated with adverse clinical characteristics, including disease stage and differentiation grade. In summary, EMH expression was common in HNSCC patients and negatively influenced their prognosis. Future studies should investigate the effect of hypoxia-modified treatment schedules in patients with high EMH expression. These may include ARCON, treatment with nimorazole, or novel targeted therapies directed at hypoxic tissue. Also, the feasibility of surgical removal of the hypoxic tumor volume prior to radiotherapy should be investigated.

Introduction

Despite improvement of surgical and radiotherapeutic techniques, as well as the introduction of systemic therapies including cisplatin or cetuximab, 5-year survival rates for patients with head and neck squamous cell carcinoma (HNSCC) remain low.[1] Currently, staging and treatment selection is based only on clinical staging using the AJCC TNM-classification. However, awareness increases that not all squamous cell carcinomas are the same, but have different tumor biology.[2] These differences could have an even greater impact on treatment outcome than mere clinical staging. An example is infection with the human papillomavirus (HPV) in oropharyngeal squamous cell carcinoma (OPSCC): HPV-associated (HPV+) OPSCC cancers show a much better response to radio- and chemotherapy than non-HPV-associated (HPV-) OPSCC.[3,4] In this line there is a clear need for other novel biomarkers to predict sensitivity to a particular treatment modality, or to identify which patients might benefit from adjuvant therapies.

One possible target or prognosticator is tumor hypoxia. Hypoxia is defined as a mismatch between cellular oxygen demand and supply. The causes of hypoxia can roughly be divided into two categories: acute or chronic hypoxia. Acute, or perfusion-limited, hypoxia, occurs when there is insufficient oxygen supply to cells due to compromise of the supplying blood vessels. Acute hypoxia causes electrolyte imbalances and an increase in intracellular hydrogen sulfide. When this occurs in specialized hypoxia-sensing cells, such as glomus or smooth muscle cells, this leads to a systemic response, such as vasodilation.[5] In contrast, chronic hypoxia triggers a cellular response in individual cells only after several hours of hypoxia.[6] Chronic hypoxia is often caused by diffusion-limitations which occur when the distance from a cell to the nearest blood vessel is too large for adequate cellular oxygenation.[7] Because of expansive tumor growth, chronic hypoxia often occurs in solid tumors, including HNSCC.[8] HNSCC is often treated with radiotherapy which depends on oxygen for free radical formation to induce DNA strand breaks and cell death. Because of the need for oxygen, tumor hypoxia causes decreased sensitivity to radiotherapy. Separately, hypoxia is thought to induce tumor progression and a more aggressive phenotype.[9] Therefore, the hypoxic status of a tumor could possibly contribute in identifying the treatment option that offers the best prognosis to an individual patient. For example, surgical removal of the hypoxic component before radiotherapy might be preferable above radiotherapy alone, when reduced sensitivity to primary radiotherapy is expected. Alternatively, hypoxia-sensitizing radiotherapy schedules such as accelerated radiotherapy with carbogen-breathing and nicotinamide (ARCON) or addition of a hypoxia-sensitizing drug like nimorazole may be considered.[10,11]

Several ways of assessing tumor hypoxia have been described.[12] This includes the invasive Eppendorf pO2 histography, that uses polarographic needles to measure tissue pO2 in vivo. Tissue biomarkers for hypoxia may also be used to assess tumor hypoxia histologically. The

use of exogenous biomarkers, for instance of the nitroimidazole class, is also invasive, as they have to be administered to patients intravenously before excision of the tissue. Finally, various endogenous biomarkers for hypoxia (EMHs) exist, that can be used to assess the hypoxic state using immunohistochemistry (IHC), with no need for additional invasive procedures other than routine diagnostic biopsy. The most important endogenous biomarkers are part of the Hypoxia-inducible factor 1(HIF-1) pathway. HIF-1 is upregulated under hypoxia to improve cellular survival in a hypoxic microenvironment. This basic helix-loop-helix transcription factor consists of an alpha (HIF-1a) and beta (HIF-1b or ARNT) subunit. Both are constitutively expressed, but under normoxic conditions HIF-1a is quickly degraded by hydroxylation and binding to the VHL protein.[13,14] In the hypoxic state, hydroxylation of HIF-1a is inhibited, causing stabilization, enabling interaction with HIF-1b and increased transcription of its downstream targets. Another EMH is osteopontin (OPN), which is expressed independently of HIF-1a and is involved in the adhesive cell–matrix interaction and is considered a protein involved in tumor development and progression.[15,16] A brief review of the studied EMHs is shown in **Box 1**.

Biomarker	Role
HIF-1a	HIF-1alpha is the alpha subunit of the HIF-1 transcription factor, which is part of the cellular defense mechanisms to survive in a hypoxic state. Under normoxic conditions it is quickly degraded by prolyl hydroxylase (PHD) 1–3. Under hypoxic conditions, PHD activity is inhibited, causing overexpression of HIF-1a. As a transcription factor, HIF-1 stabilization causes increased transcription of its downstream targets through hypoxia-responsive elements (HRE) in the DNA
HIF-2a	HIF-2alpha is also a transcription factor in the HIF family, but has distinct other downstream targets. HIF-2a stabilization under hypoxia occurs through the same mechanism as HIF-1a
CA-IX	As hypoxic cells rely on anaerobic metabolism, intracellular pH will drop because of lactate formation. Carbonic anhydrase (CA) IX is a downstream target of HIF-1 involved in pH regulation.[17]
GLUT-1	A downstream target of HIF-1a. In hypoxic conditions, additional glucose is required for the anaerobic metabolism. There are many members in the glucose transporter (GLUT-) family, but GLUT-1 is specifically upregulated by HIF-1a
OPN	Osteopontin (OPN) is an integrin-binding protein of the SIBLING family (small integrin-binding ligand N-linked glycoprotein) and was first discovered in bone tissue. It promotes cellular survival through the NF-κb pathway by reducing cell peroxide levels.[18] It is upregulated independent of HIF-1a under hypoxia

Box 1 | Endogenous markers of hypoxia

Several (narrative) reviews are currently available on the effect of HIF-1a expression on local control and survival in patients with HNSCC. However, to our knowledge, no systematic reviews have studied EMH expression from a clinical approach, by systematically comparing the effect of all EMHs according to treatment outcome and taking into account differences between subsites. In the present study, we investigate which biomarkers are used to determine tumor hypoxia in HNSCC, as well as the effect of overexpression on clinical outcome.

Methods

Search strategy

A systematic review was performed in PubMed/MEDLINE and EMBASE. The search strategy is shown in Table S1. Briefly, a search was performed for studies that described the domain ("HNSCC") and the determinant ("hypoxia"/ EMHs) or synonyms of these terms in the title or abstract or as MeSH terms. The MEDLINE GENE database was used to identify synonyms of the various EMHs. Abstracts were screened based on predetermined in- and exclusion criteria by two authors independently (**Figure 1**). Full-text analysis of potentially relevant abstracts was performed and a final selection was made. At all stages, differences were resolved by discussion. The review was limited to EMHs that were studied in more than two articles. Relevant full text papers were appraised for risk of bias using the Quality in Prognosis Studies (QUIPS) tool, that has been developed for systematic appraisal in studies of prognostic factors.[19] Using QUIPS, a risk of bias is determined, based on the study design and the reported results. For each of the six domains within QUIPS, the risk of bias was judged low (0 points), moderate (1 point) or high (2 points), based on three to seven predefined criteria per domain. For the current review, the following criteria were used: the source population should consist of a consecutive cohort of patients. Baseline characteristics should include T- and N-staging, as well as the treatment modality. Studies disclosing loss to follow-up and that confirmed whether censored patients were known to be alive at the moment of analysis were valued highest in the "study attrition" appraisal. In the correction for confounding appraisal, studies that investigated potential confounding effects of T- and N-staging, as well as treatment modality were valued highest. Finally, studies that scored a low risk of bias (≤3) were included.

Data extraction and meta-analysis

Extracted data included number of patients, disease stage, tumor subsite, treatment, biomarkers, and corresponding cutoffs and outcome. The studied outcomes were the hazard ratios (HR) for locoregional control (LRC), overall survival (OS), disease-free survival (DFS) and disease-specific survival (DSS). If a HR was not described, but a Kaplan–Meier curve was

available, the curve was digitized using the open-source Engauge Digitizer software (http://digitizer.sourceforce.net) and a univariate HR was estimated through the methods of Tierney et al.[20] Meta- analysis was considered only if studies used the same cutoff values for EMH positivity and described patient cohorts were comparable in terms of treatment and disease stage. A review protocol was not previously published. Results are presented in accordance with the PRISMA statement for systematic reviews.[21]

Results

Study selection

The search in EMBASE and PubMed yielded 4684 unique publications. Abstract screening yielded 138 potentially interesting papers, of which the full text was requested. Of these papers, 17 were not in English, Dutch or German, 19 were conference abstracts with no full text paper available and 36 were excluded for various reasons of mismatch with the domain, determinant, or outcome. Sixty-six papers remained for critical appraisal. Relevant reviews

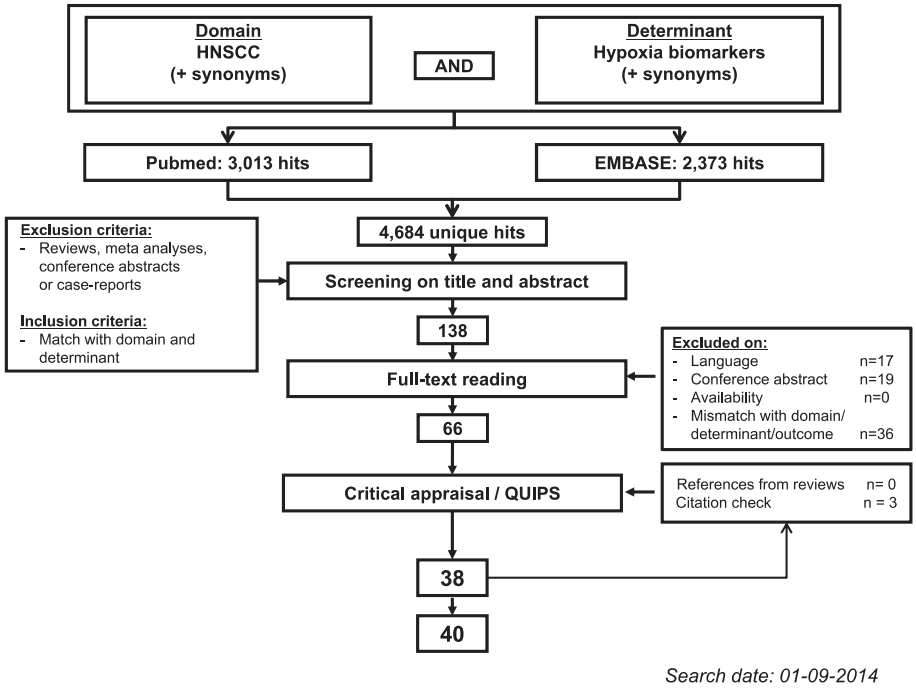

Figure 1 | Study selection process. Study selection flowchart. Of the 66 suitable papers, 38 were found of adequate quality. A citation check yielded 3 additional results, of which 2 were of adequate quality. In total, 40 studies were included.

were read, references were screened and a citation check was performed using Web of Science. This yielded three additional papers. The study selection process is shown in **Figure 1**.

Critical appraisal

Using the QUIPS criteria, the 66 and 3 papers identified through the search and citation checks, respectively, were appraised (Table 1 for included studies, Table S2 for excluded studies). In many papers it was not described whether the cohort was consecutive. Also, loss to follow-up and the characteristics of patients lost to follow-up were rarely reported, resulting in a high risk of bias in the "Study Attrition" domain. Many studies used data-dependent cutoffs for their prognostic factor assessment, scoring lower on the "Prognostic factor" domain. Also, the treatment modality was often not included in multivariate analysis. As the effect of hypoxia might be different for each treatment modality (e.g., surgery, radiotherapy, or chemoradiation [CRT]), these papers scored a higher risk of bias in the "Confounding" domain, except when the cohort received uniform treatment. A score was calculated as described before, however, the study attrition score was omitted in this final risk of bias score. Forty papers remained for final analysis.

Endogenous markers of hypoxia

The included studies described the effect of EMH expression across all subsites in the head and neck area. However, many studies analyzed different staining patterns, methods, and cutoff values to define EMH positivity. For instance, some studies used an H-score that combined staining proportion and intensity, while others only scored staining proportion or intensity. Because of this heterogeneity, it was deemed that data pooling and subsequent meta-analysis were not appropriate, as this might introduce bias. EMH expression was often correlated with adverse clinical parameters, such as T-stage, N-stage, or differentiation grade, as shown in **Tables 2 through 6**. The effect of EMH expression is discussed per treatment strategy, as hypoxia may influence outcome of various treatment strategies differently. As a summary, a forest plot for OS across all treatment strategies is shown in **Figure 2**.

Primary radiotherapy or ARCON

Eleven studies were identified that studied the clinical effect of EMHs in patients treated with radiotherapy (XRT) or the hypoxia-sensitizing treatment of accelerated radiotherapy, in combination with carbogen gas breathing and intravenously administered nicotinamide (ARCON).[22,23,32,24–31] Results are summarized in **Table 2**. Most studies identified a worse outcome in patients with high EMH expression. This finding appears to be present across all subsites within the head and neck region, including the oropharynx,[22,23] the larynx,[25,27] and

Study	SP	SA	PF	O	C	AR	B
Aebersold 2011	L	H	L	L	M	L	1
Avirovic 2013	M	L	M	L	M	L	3
Brockton 2012	L	H	M	L	M	L	2
Brockton 2011	M	H	M	L	M	L	3
Cabanillas 2009	H	H	L	L	M	L	3
Chien 2008	H	H	L	L	L	M	3
Chien 2009	M	L	L	L	M	L	2
Choi 2014	M	H	L	L	M	M	3
Choi 2008	M	L	L	L	M	M	3
Dos Santos 2012	M	L	L	L	L	M	2
Douglas 2013	L	H	L	L	L	L	0
Dunkel 2013	L	H	L	L	M	M	2
Eckert 2010	H	L	L	L	M	L	3
Eckert 2011	H	L	L	L	L	L	2
Eriksen 2007	L	L	L	L	L	L	0
Fillies 2005	H	H	M	L	L	L	3
Grimm 2014	H	L	L	L	M	L	3
Hong 2013	L	L	L	L	L	L	0
Hui 2002	L	H	L	L	L	L	0
Jonathan 2006	M	L	L	L	L	L	1
Kim 2007	H	L	M	L	L	L	3
Kitagawa 2013	H	L	M	L	L	L	3
Kwon 2014	H	L	L	L	M	L	3
Le 2007	H	L	M	L	L	L	3
Liang 2011	L	H	L	L	H	L	2
Nordsmark 2007	M	L	L	L	M	M	3
Pérez-Sayans 2012	M	L	L	L	M	M	3
Rademakers 2013	M	L	M	L	L	L	2
Rahimi 2012	L	H	L	L	L	L	0
Roh 2009	L	L	M	L	M	L	2
Schrijvers 2008	L	L	M	L	L	L	1
Silva 2008	M	H	L	L	M	L	2
v.d. Broek 2009	L	H	M	L	L	L	1
Wachters 2013	L	H	L	L	L	L	0
Wan 2012	M	H	M	L	L	L	2
Wildeman 2009	M	L	L	L	M	L	2
Winter 2006	M	L	L	L	M	L	2
Xueguan 2008	L	L	L	L	M	M	2
Zheng 2013	M	H	L	L	M	M	3
Zhu 2010	L	H	M	L	L	L	1
Totals							

Table 1 | Critical appraisal and description which biomarkers is described in the study. SP: study participation; SA: study attrition; PF: prognostic factor; O: Outcome; C: Confounding; AR: Statistical analysis and reporting. B: Bias score according to QUIPS. Low = 0, Moderate = 1, High = 2 points. SA was not included. Studies with a bias score >3 were excluded.

Study	HIF-1a	HIF-2a	CA-IX	GLUT-1	OPN
Aebersold 2011	•				
Avirovic 2013					•
Brockton 2012			•		
Brockton 2011			•	•	
Cabanillas 2009	•				
Chien 2008					•
Chien 2009					•
Choi 2014	•				
Choi 2008			•		
Dos Santos 2012	•				
Douglas 2013	•		•		
Dunkel 2013	•				
Eckert 2010	•		•		
Eckert 2011	•			•	
Eriksen 2007			•		
Fillies 2005	•				
Grimm 2014				•	
Hong 2013	•				
Hui 2002	•		•		
Jonathan 2006			•	•	
Kim 2007			•		
Kitagawa 2013	•				
Kwon 2014	•		•	•	
Le 2007			•		•
Liang 2011	•	•			
Nordsmark 2007	•		•		•
Pérez-Sayans 2012			•		
Rademakers 2013			•		
Rahimi 2012	•		•		
Roh 2009	•		•	•	
Schrijvers 2008	•		•	•	
Silva 2008	•				
v.d. Broek 2009	•		•		
Wachters 2013	•		•		•
Wan 2012	•				
Wildeman 2009	•		•		
Winter 2006	•	•	•		
Xueguan 2008	•				
Zheng 2013	•				
Zhu 2010	•	•			
Totals	27	3	21	7	6

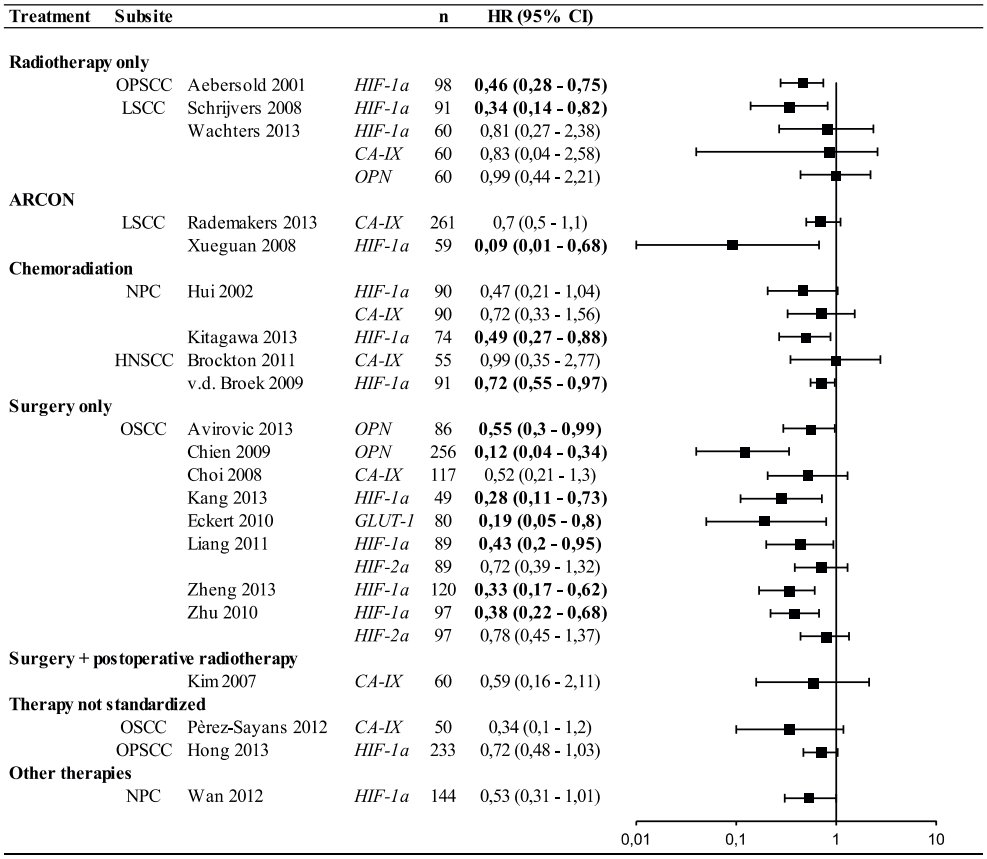

Figure 2 | Forest plot: Overall survival and EMH Expression. Visual summary of studies that described overall survival. ARCON: accelerated radiotherapy, carbogen gas and nicotinamide. HRs < 1 indicate beneficial prognosis for non-hypoxic tumors. Therapy not standardized: All treatment modalities were analyzed in a single cohort. Data were not presented according to therapy. The studies of Pérez-Sayáns and Hong describe their entire cohort of patients, receiving any treatment. In the study of Wan patients were randomized between neoadjuvant radiotherapy or chemoradiation, followed by concurrent chemoradiation.

the nasopharynx,[30] as well as in a study that analyzed patients with cancers of several subsites.[31] In this last study multiple biomarkers of hypoxia were studied. HIF-1a expression predicted significantly worse LRC. While LRC was lower in patients with high expression of CA-IX and OPN, this did not reach statistical significance. The study of Rademakers *et al* of 261 patients randomized between treatment with XRT or ARCON did not find better OS in low CA-IX expressing laryngeal cancer patients (LSCC).[26] Unfortunately, no separate data were presented for XRT and ARCON. The authors did report differences in OS between different staining patterns: a perinecrotic staining pattern, in which cells stain more strongly as the distance to the nearest blood vessel increases, was associated with worse OS (P <

0.01) and LRC (P = 0.01) when compared to diffuse or no expression of CA-IX. Surprisingly, in the study of Jonathan *et al* of 58 HNSCC patients treated only with ARCON, a better outcome was observed in patients with high EMH expression.[32] In this small study, CA-IX expression was mostly low. Using a cutoff at the median value of expression, the authors describe no significant correlation with the outcome. Finally, the 80th percentile (25% membranous expression) was used as a cutoff value that found a significantly better outcome for patients with high CA-IX levels.

Primary CRT

Only four studies were available that studied EMH expression in a cohort of patients treated with CRT only.[33–36] A significant effect of EMH expression on survival was found in two.[34,36] Kitagawa *et al* described a cohort of 74 nasopharyngeal cancer (NPC) patients treated with CRT (with exempt of seven patients that did not receive chemotherapy because of kidney failure), patients with more than 10% HIF-1a expression had significantly worse OS. Van den Broek *et al* studied HIF-1a expression in a cohort of 91 HNSCC patients and described worse OS, but not LRC in patients with higher HIF-1a expression. Hui *et al* studied a cohort of 90 NPC patients and found a trend toward better outcome in low HIF-1a-expressing patients, but did not find a similar trend for CA-IX. Brockton *et al* studied CA-IX and GLUT-1 expression in a smaller cohort of 58 patients with primary tumors from various subsites in the head and neck and also did not find a correlation with survival. Results are summarized in **Table 3**.

Primary surgery

Thirteen studies studied EMH expression in patients treated with primary surgery only.[37,38,47–49,39–46] All studies concerned oral cavity carcinoma (OSCC) and all studies, except two, described that in surgically treated patients, HIF-1a expression significantly decreased the prognosis. Choi *et al* investigated CA-IX expression in a cohort of 118 patients and did not find an association with prognosis. The study of Dos Santos *et al* describes a small subgroup of 36 patients treated only with surgery in a larger cohort of 66 OSCC patients and did not find a difference between high and low HIF-1a-expressing patients. Results are summarized in **Table 4**.

Surgery and postoperative radiotherapy

Eight studies were available that studied EMH expression in a cohort of patients treated with surgery and postoperative radiotherapy.[41,50–56] Surprisingly, two studies described better prognosis in high HIF-1a expressing patients.[41,51] The study of Fillies *et al* described the effect of HIF-1a expression on clinical outcome and found that HIF-1a expression above 5% results in better survival in OSCC patients. The study of Dos Santos *et al* describes a small subgroup of 30 patients treated with surgery and postoperative radiotherapy within a study

Study	treatment	stage	EMH	Pos/n	Cutoff	correlations
Oropharyngeal carcinoma						
Aebersold 2001	XRT	Any	HIF-1a	92/98	10% N	Tumor grade
Silva 2008	XRT		HIF-1a	43/79	10%	Low Hb
Laryngeal carcinoma						
Douglas 2013	XRT	I-II	HIF-1a	124/271	10% N	None
Kwon 2014	XRT	I-II	HIF-1a	7/42	50% N	ns
		I-II	CA-IX	17/42	30% M	ns
Rademakers 2013	ARCON/XRT*	III-IV	CA-IX	132/261	Med†	None
Schrijvers 2008‡	XRT	I-II	HIF-1a	46/91	0.5% N	None
		I-II	CA-IX	39/91	12.5% M	None
		I-II	GLUT-1	53/91	35% M	ns
Wachters 2013	XRT	I-II	HIF-1a	47/60	0.5% N	None
		I-II	CA-IX	11/60	12.5% M	None
		I-II	OPN	20/60	0.5% C	ns
Wildeman 2009	XRT	Any	HIF-1a	59	N/M %§	ns
		Any	HIF-1a	59	Int	ns
		Any	CA-IX	59	int	ns
Nasopharyngeal carcinoma						
Xueguan 2008	ARCON	Any	HIF-1a	40/59	10% N	None
Multiple subsites						
Nordsmark 2007	XRT	Any	HIF-1a	19/59**	50% N	ns
			CA-IX	26/57**	10% M	ns
			OPN	17/57	Int D	ns
Jonathan 2006	ARCON	Any	CA-IX	29/58	25% M	ns
			GLUT-1	29/58	Int D	ns

Table 2 | Clinical outcome: Radiotherapy / ARCON. The outcomes locoregional control (LRC), overall survival (OS), disease-free survival (DFS) and disease-specific survival (DSS) are shown as hazard ratio (95% confidence interval). Hazard ratios < 1 indicate beneficial prognosis for non-hypoxic tumors. Significant values are shown in bold. Cutoff: EMHs were scored according to nuclear (N), membranous (M), cytoplasmic (C) or diffuse (D) staining patterns. Int: staining intensity was scored. XRT: radiotherapy. ARCON: accelerated radiotherapy, carbogen gas breathing and nicotinamide. Pos: number of patients with positive staining. LR : Logrank test. ns: not specified. Multiple subsites: patients were not analyzed per subsite.

* Patients were randomized between ARCON and XRT.

† Computerized image analysis was performed and the median value was used in statistical analyses.

‡ Supraglottic carcinomas only

§ Analyses were performed using the proportion of membranous or nuclear staining cells as a continuous variable, no cutoff was used. Number of positive staining patients is therefore not relevant.

** Data on immunohistochemical analysis were available for 59/67 patients (HIF-1a) and 57/67 patients (CA-IX)

Study	LRC	OS	DFS	DSS
Oropharyngeal carcinoma				
Aebersold 2001		**0.46 (0.28 – 0.75)**	**0.50 (0.30 – 0.83)**	
Silva 2008	**0.2 (0.1 - 0.42)**			
Laryngeal carcinoma				
Douglas 2013	0.96 (0.79 – 1.16)			LR p=0.23
Kwon 2014	**0.13 (0.02 – 0.82)**			
	0.11 (0.01 – 0.96)			
Rademakers 2013		0.7 (0.5 – 1.1)		
Schrijvers 2008††		**0.34 (0.14 - 0.82)**		
	0.34 (0.14 - 0.85)	ns		
	ns			
Wachters 2013	0.93 (0.26 - 3.45)	0.81 (0.27 - 2.38)		
		0.83 (0.04 – 2.58)		
		0.99 (0.44 – 2.21)		
Wildeman 2009	1.08 (0.91 -1.29)‡‡			
	0,92 (0,56 - 1,49)‡‡			
	1.21 (0.96 – 1.52) ‡‡			
Nasopharyngeal carcinoma				
Xueguan 2008	0.41 (0.06 – 2.69)	**0.09 (0.01 – 0.68)**§§	**0.26 (0.07 – 0.97)**	
Multiple subsites				
Nordsmark 2007	**0.22 (0.06 - 0.81)**			
	0.35 (0.12 – 1.01)			
	0.83 (0.35 – 2.00)			
Jonathan 2006	**4.23 (1.07 – 16.76)**§§	ns		
	ns	**LR p=0.001**		

†† Supraglottic carcinomas only

‡‡ Presented numbers are odds-ratios for 2-year locoregional recurrence, not hazard ratios

§§ Last surviving patient was scored as deceased to enable HR-calculation, because of 100% survival in one arm.

of 66 OSCC patients. The study of Winter et al. describes significantly worse outcome for HNSCC patients with high HIF-1a expression. All other studies did not show a difference in outcome when patients were stratified according to EMH expression. Results are summarized in **Table 5**.

Other treatment strategies

Seven studies describe data from larger cohorts treated with various treatment modalities, depending on localization and staging.[57–63] Only the studies of Rahimi and Le identified a significant correlation between EMH expression and outcome. Rahimi *et al* found significant better DFS and improved, but not significantly different LRC for patients expressing no or very low levels (<1%) of HIF-1a [60]. Similar results were not obtained for CA-IX. Le *et al* studied both CA-IX and OPN expression and found better survival in patients with low CA-IX expression. Pérez-Sayáns *et al* (CA-IX), Hong *et al* (HIF-1a), and Choi *et al* (HIF-1a) did not find a correlation with outcome. Wan *et al* studied 144 NPC patients randomized to receive neoadjuvant radiotherapy and neoadjuvant CRT followed by CRT and found better, although not statistically significant, OS in low HIF-1a expressing patients. The same results were obtained when both treatment arms were analyzed separately. Results are summarized in **Table 6**.

Study	stage	EMH	Pos/n	Cutoff	correlations	LRC	OS
Nasopharyngeal cancer							
Hui 2002	III-IV	HIF-1a	32/90	5% N	None		0.47 (0.21 - 1.04)
		CA-IX	32/90	5% M	None		0.72 (0.33 - 1.56)
Kitagawa 2013	Any	HIF-1a	27/74*	10% N	None		**0.49 (0.27 - 0.88)**
Multiple subsites							
Brockton 2011	II-IV	CA-IX	23/46†	Med‡	None		0.99 (0.35 - 2.77)
		GLUT-1	24/47†	Med	None		LR p=0.79
v.d. Broek 2009	Any	HIF-1a	91	N/M§	ns	0.64 (0.36 - 1.12)	**0.72 (0.55 - 0.97)**
		CA-IX	91	M/C§	ns	0.73 (0.43 – 1.23)	ns

Table 3 | Clinical outcome: Primary chemoradiation. The outcomes locoregional control (LRC), overall survival (OS) are shown as hazard ratio (95% confidence interval). There were no data on disease free survival (DFS) or disease specific survival (DSS) and these columns are not shown. Hazard ratios < 1 indicate beneficial prognosis for non-hypoxic tumors. Significant values are shown in bold. Cutoff: EMHs were scored according to nuclear (N), membranous (M), cytoplasmic (C) or diffuse (D) staining patterns. Pos: number of patients with positive staining. Int: staining intensity was scored. LR: Logrank test. Ns: not specified. Multiple subsites: patients were not analyzed per subsite.

* 7 patients received radiotherapy only due to kidney failure
† Total 55 patients, data were missing because of missing or folded TMA cores
‡ Computerized image analysis was performed, staining pattern was not taken into account
§ Nuclear or membranous expression was analyzed as a continuous variable

Study	stage	EMH	Pos/n	Cutoff	correlations
Oral cavity					
Avirovic 2013	Any	OPN	48/86	71% C	N-stage, disease stage
Chien 2008	Any	OPN	30/94	10% C	T-stage, N-stage, tumor thickness, tumor necrosis
Chien 2009*	Any	OPN	192/256††	10% C	T-stage, N-stage, disease stage
Choi 2008	Any	CA-IX	64/117	5% M	None
Dos Santos 2012	Any	HIF-1a	15/36	6/9†	None
Dunkel 2013	I	HIF-1a	16/44	Int	ns
Kang 2013†	I-III	HIF-1a	43/49	10% ‡	T-stage, N-stage, tumor grade
Eckert 2010	Any	HIF-1a	80§	3-4 vs. 6-8 C** 3-4 vs. 9-12 C**	T-stage
Eckert 2011	Any	GLUT-1	80††	0-2 vs 3-4 M** 0-2 vs 6-8 M** 0-2 vs9-12 M**	None
Grimm 2014	Any	GLUT-1	161	50% M/C‡‡	ns
Liang 2011	Any	HIF-1a	89	25% N/C‡‡	Tumor grade, N-stage
		HIF-2a	89	25% N/C‡‡	T-stage
Zheng 2013	Any	HIF-1a	120	1% N	N-stage, disease stage
Zhu 2010	Any	HIF-1a	97	1% N	T-stage, N-stage, tumor grade
		HIF-2a	97§	1% N	T-stage, microvessel density

Table 4 | Clinical outcome: Primary surgery. The outcomes locoregional control (LRC), overall survival (OS), disease-free survival (DFS) and disease-specific survival (DSS) are shown as hazard ratio (95% confidence interval). Hazard ratios < 1 indicate beneficial prognosis for non-hypoxic tumors. Significant values are shown in bold. Cutoff: EMHs were scored according to nuclear (N), membranous (M), cytoplasmic (C) or diffuse (D) staining patterns. Pos: number of patients with positive staining. Int: staining intensity was scored. LR: Logrank test. ns: not specified. Multiple subsites: patients were not analyzed per subsite.

* Patients from Chien 2008 were also included in the sample from Chien 2009

† A score range 0-9 was calculated based on staining proportion and intensity. Staining pattern was not disclosed

‡ The scored staining pattern was not disclosed

§ Negative expression (score 0-2): 11, weak (3-4): 24, moderate (6-8) 38, strong (9-12): 7 patients.

** A score range 0-12 was calculated based on staining proportion and intensity.

†† Negative expression (score 0-2): 32, weak (3-4): 13, moderate (6-8) 21, strong (9-12): 11 patients.

‡‡ Both membranous and cytoplasmic (M/C) or nuclear and cytoplasmic (N/C) staining cells were scored positive

Study	EMH	OS	DFS	DSS
Oral cavity				
Avirovic 2013	OPN	**0.55 (0.3 - 0.99)**		
Chien 2008	OPN		**LR p<0.001**	
Chien 2009*	OPN	**0.12 (0.04 - 0.34)**		
Choi 2008	CA-IX	0.52 (0.21 – 1.30)		
Dos Santos 2012	HIF-1a			LR p=0.7
Dunkel 2013	HIF-1a		**LR p=0.022**	LR p=0.29
Kang 2013‡	HIF-1a	**0.28 (0.11 – 0.73)**	**0.34 (0.15 – 0.79)**	
Eckert 2010	HIF-1a	**0.21 (0.06 – 0.72)** **0.19 (0.05 – 0.80)**		
Eckert 2011	GLUT-1			0.71 (0.20 – 2.54) **0.29 (0.12 – 0.71)** 0.5 (0.15 – 1.63)
Grimm 2014	GLUT-1			**0.58 (0.37 - 0.91)**
Liang 2011	HIF-1a	**0.43 (0.20 – 0.95)**		
	HIF-2a	0.72 (0.39 - 1.32)		
Zheng 2013	HIF-1a	**0.33 (0.17 - 0.62)**	**0.30 (0.16 - 0.75)**	
Zhu 2010	HIF-1a	**0.38 (0.22 - 0.68)**	**0.44 (0.26 - 0.75)**	
	HIF-2a	0.78 (0.45 - 1.37)	0.87 (0.51 - 1.47)	

* Patients from Chien 2008 were also included in the sample from Chien 2009

Study	stage	EMH	Pos/n	Cutoff	correlations	favor
Oral cavity						
Brockton 2012	Any	CA-IX	17/61	p75*	ns	L
Dos Santos 2012	Any	HIF-1a	16/30†			H
Fillies 2005	I-II	HIF-1a	45/85	5% N	None	H
Han 2012	II	HIF-1a	4/33	10% N	ns	L
Kim 2007	Any	CA-IX	38/60	10% C/M	Tumor grade, subsite, smoking	L
Roh 2009	II	HIF-1a	6/43	1% N	None	L
		CA-IX	26/43	10% M	Tumor thickness	L
		GLUT-1	31/43	50% M	Tumor thickness, N-stage	L
Laryngeal cancer						
Cabanillas 2009	Any	HIF-1a	75/106	10% N	T-stage, disease stage	H
Multiple sites						
Winter 2006	Any	HIF-1a	45/151	Med N‡	Disease stage	L
		HIF-2a	21/151	Med N/C‡	None	L
		CA-IX	92/151	Med M‡	ns	-

Table 5 | Clinical outcome: Primary surgery + postoperative radiotherapy. The outcomes locoregional control (LRC), overall survival (OS), disease-free survival (DFS) and disease-specific survival (DSS) are shown as hazard ratio (95% confidence interval). Hazard ratios < 1 indicate beneficial prognosis for non-hypoxic tumors. Significant values are shown in bold. Cutoff: EMHs were scored according to nuclear (N), membranous (M), cytoplasmic (C) or diffuse (D) staining patterns. Pos: number of patients with positive staining. LR: Logrank test. ns: not specified. Multiple subsites: patients were not analyzed per subsite.

* Computerized image analysis, scoring pattern and value not disclosed.

† Subgroup within a larger study of 66 patients

‡ Exact value not disclosed

Study	EMH	LRC	OS	DFS	DSS
Oral cavity					
Brockton 2012	CA-IX				0.26 (0.06 – 1.05)
Dos Santos 2012	HIF-1a				**3.41 (1.13 – 10.34)**
Fillies 2005	HIF-1a		**LR p<0.05***	**LR p=0.02**	
Han 2012	HIF-1a				
Kim 2007	CA-IX		0.59 (0.16 - 2.11)	0.85 (0.33 - 2.23)	
Roh 2009	HIF-1a	LR p=0.154			0.37 (0.13 – 1.03)
	CA-IX	LR p=0.857			LR p=0.159
	GLUT-1	LR p=0.416			LR p=0.060
Laryngeal cancer					
Cabanillas 2009	HIF-1a		LR p=0.8		LR p=0.5
Multiple sites					
Winter 2006	HIF-1a		LR p=0.08	**LR p=0.02**	**LR P = 0.02**
	HIF-2a		LR p=0.43	LR p=0.10	LR P = 0.16
	CA-IX		LR p =0.3	LR p=0.2	LR p=0.1

* Exact value not disclosed

Study	treatment	stage	EMH	Pos/n	Cutoff	correlations
Oral cavity						
Pérez-Sayans 2012	Any	Any	CA-IX	23/50[*]	50% M	Disease stage
Oropharyngeal cancer						
Hong 2013	Any	Any	HIF-1a	137/233	10% N	T-stage, tumor grade
Rahimi 2012	XRT/CRT[†]	Any	HIF-1a	26/58	1% N	ns
			HIF-1a	NS/58	1%C	
		Any	CA-IX	NS/57	1%C	ns
			CA-IX	NS/57	1%M	
Wan 2012	nC+R/ nC+CRT[‡]	Any	HIF-1a	66/144*	5/16 C/N[§]	ns
Multiple subsites						
Choi 2014	Any	Any	HIF-1a	25/76	1% C	None
Eriksen 2007	[**]	Any	CA-IX	370	M[††]	None
Le 2007	Any	Any	CA-IX	29/94[‡‡]	Int C[§§]	ns
		Any	OPN	70/84	Int D	ns

Table 6 | Clinical outcome: Other treatment strategies. The outcomes locoregional control (LRC), overall survival (OS), disease-free survival (DFS) and disease-specific survival (DSS) are shown as hazard ratio (95% confidence interval). Hazard ratios < 1 indicate beneficial prognosis for non-hypoxic tumors. Significant values are shown in bold. Cutoff: EMHs were scored according to nuclear (N), membranous (M), cytoplasmic (C) or diffuse (D) staining patterns. Pos: number of patients with positive staining. Int: staining intensity was scored. LR: Logrank test. ns: not specified. Multiple subsites: patients were not analyzed per subsite

[*] 23 patients had intense staining, 18 patients had moderate staining and 9 patients had no staining.

[†] Chemotherapy was added in the case of T4 or N3 disease.

[‡] Patients participated in a RCT between neoadjuvant chemotherapy and either radiotherapy or chemoradiation

[§] A score was calculated from 0-16, based on staining proportion and intensity. Both cytoplasmic and nuclear patterns were scored.

[**] Patients were randomized between radiotherapy or radiotherapy and the radiosensitizer nimorazole

[††] Patients were analyzed in groups: <1%, 1-10%, 10-30% and above 30%. None of these subgroups showed significant better improval compared to the other groups

[‡‡] Results for CA-IX and OPN were available for 94 and 84 patients, respectively, because of TMA core availability.

[§§] Expression was scored as negative, weak or strong by a single pathologist.

Study	EMH	LRC	OS	DFS	DSS
Oral cavity cancer					
Pérez-Sayans 2012	CA-IX		0.34 (0.1 - 1.2)*		
Oropharyngeal cancer					
Hong 2013	HIF-1a		0.72 (0.48 – 1.03)		0.75 (0.46 – 1.22)
Rahimi 2012	HIF-1a	0.76 (0.55 – 1.01)		0.81 (0.67 – 0.99)	
	HIF-1a	1.10 (0.72 – 1.67)		1.06 (0.83 – 1.35)	
	CA-IX	1.52 (0.71 - 3.23)		1.03 (0.81 - 1.32)	
	CA-IX	0.93 (0.77 - 1.12)		1.01 (0.88 - 1.15)	
Wan 2012	HIF-1a		0.53 (0.31 - 1.01)		
Multiple subsites					
Choi 2014	HIF-1a		LR p=0.237	0.55 (0.33 - 1.15)	
Eriksen 2007	CA-IX	LR p=0.8			
Le 2007	CA-IX		LR p=0.011		LR p=0.030
	OPN		p>0.05		

* Strong vs. no CA-IX staining.

Discussion

In this systematic review, we investigated expression of biomarkers for tumor hypoxia in relation to clinical outcome and treatment strategy. We identified 40 high-quality studies. EMH expression was common and associated to worse survival or LRC in most studies, although statistical significance was not always reached. In addition, EMH expression was often correlated with worse clinicopathological characteristics. Surprisingly, three studies found EMH expression to be associated to better outcome, but these mostly had a small sample size.[32,41,51] Moreover, in studies that investigated multiple EMHs, high HIF-1a expression was often associated with worse outcome, while this was not always true for the other EMHs. Chronic hypoxia is an important and highly prevalent problem in solid tumors.[9] We observed that several adverse clinical parameters were often associated with higher EMH expression, such as the presence of cervical lymph node metastasis, higher T-stages, and worse differentiation grade. The latter two correlations support the hypothesis that hypoxia occurs more often in larger and faster growing tumors. Despite the correlations to clinical parameters, the presence of hypoxia was often an independent predictor of adverse outcome. One explanation might be that in the hypoxic microenvironment, several mechanisms are activated that improve cellular survival under these adverse circumstances. As the HIF-1a transcription factor is stabilized, transcription of proteins increases, including those involved in pH regulation (CA-IX), cellular metabolism (GLUT-1), but also genes involved in angiogenesis or oxygen transport. OPN expression occurs through a hypoxia-dependent, HIF-independent pathway and reduces cell death and apoptosis in hypoxic or reoxygenated cells, it may therefore signify a more aggressive tumor phenotype.[15,16,64,65]

Radiotherapy relies on the formation of free oxygen radicals to induce DNA strand breaks and cell death.[66] Also, radiotherapy causes apoptosis through stabilization of p53. The HIF-1 pathway upregulates proteins involved in epithelial-to-mesenchymal transition (EMT), including the transcription factor Snail.[67,68] Radiation-induced DNA damage is reduced by EMT by emergence of cancer stem cells that express high levels of free radical-scavenging proteins.[69] Moreover, Snail causes radioresistance by suppressing p53-mediated apoptosis.[66,69] Snail also contributes to cisplatin resistance, which is often concurrently administered to patients as a radiosensitizer.[70] Thus, hypoxia does not only directly affect (chemo)radiosensitivity, but also indirectly through EMT.

In this review we identified several studies that show that increased EMH expression leads to worse LRC and survival. However, in patients that were surgically treated only, EMH expression was also associated with worse outcome.[37–39,45,47] This supports the hypothesis that hypoxia also contributes to a more aggressive tumor phenotype as described above. Surprisingly, only few studies on HNSCC patients treated with surgery and postoperative

(chemo)radiotherapy found an association between EMH expression and survival. A possible explanation is that decreased tumor volume, will lead to better sensitivity to radiotherapy, as was shown by Pameijer and Chen.[71,72] Dunst and colleagues even described that the hypoxic tumor volume is a better prognosticator than total tumor volume to predict outcome after radiotherapy.[73]

Although EMH expression has been well described in HNSCC in current literature, there are still opportunities for future studies. In many publications, EMH expression was studied using tissue microarrays (TMAs).[74] In a TMA, tumor tissues from multiple patients are placed on a single histological slide.[75] This technique allows for high throughput in determining biomarker expression in patients, but also introduces the risk of sampling bias. Unfortunately, EMH expression may vary widely within tumors, as hypoxia may occur more often in cells that are located distantly from microvessels. This will result in intratumor heterogeneity in the expression of EMH. Positive staining for hypoxic markers is often found in areas of necrosis (perinecrotic staining patterns), which is by some considered proof of "actual hypoxia". Alternatively, diffuse expression of HIF-1a may also be observed, and is thought to derive from an oncogene-driven overexpression or stabilization of HIF-1a. These expression patterns may be visualized better in whole-slide tissue sections, rather than TMAs. Unfortunately, staining patterns in whole slides have only been described in two studies. Perinecrotic CA-IX staining was associated with worse outcome in a large cohort of LSCC patients treated with ARCON.[26] In fact, staining pattern was a stronger predictor of outcome than the percentage of positive staining cells. In a smaller study of OPSCC patients, there was no difference between perinecrotic and diffuse HIF-1a staining patterns.[22]

In the past years, HPV+ HNSCC has emerged as a separate entity with a difference in tumor biology, but also a better response to therapy. There may be differences in the prevalence of tumor hypoxia in HPV+ cancers and tumor hypoxia might also affect the prognosis of HPV+ cancers differently than HPV- cancers. This should be investigated in future studies. Finally, the effect of therapies that focus on hypoxia, either through improvement of tumor oxygenation, or by targeting hypoxic tumor cells should be the subject of future studies. Promising therapies include ARCON, the combination of radiotherapy with carbogen gas breathing and nicotinamide administration, which is currently tested in phase III trials.[10,76] Delivering increased radiotherapy doses to hypoxic areas in a tumor by IMRT "dose-painting" is also considered, but not yet widely applied.[77,78] Alternatively, the hypoxia-sensitizer nimorazole may be added to primary radiotherapy.[11] The efficacy of nimorazole administration in HNSCC has been shown in trials within the DAHANCA group.[79] Finally, surgery may also be a treatment option for hypoxic tumors, to decrease the hypoxic or therapy-resistant fraction. While devascularization of the surgical field may lead to hypoxia in the direct postoperative phase, revascularization occurs as early as several days after surgery.[80] This may increase oxygenation, making remaining tumor cells more susceptible

to postoperative radiotherapy. Future studies should investigate the feasibility of such a multimodal approach and the effect on survival of patients with hypoxic tumors.

Several limitations of this review and the identified literature should be considered. In the literature, many ways to score biomarker positivity were used. Most studies scored percentages of positive staining cells, while others also took into account staining intensity, or combined the two using a H-score. If HIF-1a expression will be used in treatment selection, a validated, and reproducible scoring strategy should be employed, preferably without software imaging analysis, that may not be available in all centers. Moreover, the different cutoff points, as well as large heterogeneity in terms of tumor subsite and tumor stages did not allow for proper meta-analysis of the extracted data. As the cutoff points used in the individual studies were most often the ideal cutoff values for each individual data set, data pooling may introduce bias, and give an overestimation of the effect. Therefore, we have not performed this, in contrast to an earlier review on HIF-1a only.[81] To provide a visual overview of the results, a forest plot is provided. Another limitation of this review is that we did not include hypoxia gene expression profiles in the search. Several articles describe such profiles in head and neck cancer.[82–86] In the present study we have chosen to focus on IHC, as HNSCC is still highly prevalent in resource-limited areas. Compared to techniques like quantitative PCR (qPCR) or the use of microarrays, IHC may be performed at relatively low cost.

Conclusion

In this systematic review, we identified HIF-1a, HIF-2a, CA-IX, GLUT-1, and OPN as the best studied endogenous markers of tumor hypoxia. In general, expression of these biomarkers was associated with worse survival, almost regardless of the therapy provided. These proteins are not only biomarkers, but are also part of cellular survival mechanisms. Therefore, EMH overexpression may result in worse prognosis not only due to hypoxia, but also because of a more aggressive tumor phenotype. The effect of tumor hypoxia in HNSCC patients warrants further investigation. Studies should investigate the best treatment option for hypoxic tumors, for instance hypoxia-modified radiotherapy schedules, targeted therapies against hypoxic cells or excision of the hypoxic tissue to improve radiation sensitivity. Knowledge on the tumor hypoxia status will help clinicians to select tailored treatments for each individual patient and thus enable personalized cancer care.

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Table S1. Search strategy

	PubMed	
#1	(("upper aerodigestive tract"[tiab] OR UADT[tiab] OR (Head[tiab] OR Neck[tiab]OR "Head and neck"[Tiab] OR oropharyn*[Tiab] OR pharyn*[Tiab] OR tonsil*[Tiab] OR "alveolar process"[Tiab] OR Palate[tiab] OR oral[Tiab] OR tong*[Tiab] OR laryn*[Tiab] OR mouth[tiab] OR nasopharyn*[Tiab] OR squamous[tiab]) AND (Cance*[tiab] OR neoplasm*[tiab] OR malignan*[tiab] OR Tumo*[tiab] OR Tumou*[tiab] OR Carcinom*[tiab] OR oncolog*[tiab] OR adenocarci*[tiab])) OR "Head and Neck Neoplasms"[MeSH] OR "Carcinoma, squamous cell of head and neck"[Supplementary Concept]	*Domain*
#2	hypox*[tiab] OR HIF-1*[tiab] OR HIF1*[tiab] OR HIF-2*[tiab] OR "HIF2"[tiab] OR Oxygena*[tiab] OR "carbonic anhydrase-9" [tiab] OR "CA-9" [tiab] OR "CA-IX"[tiab] OR "carbonic anhydrase"[tiab] OR Osteopontin[tiab] OR Furin[tiab] OR "Hypoxia-Inducible Factor 1"[MeSH] OR "Cell Hypoxia"[MeSH] OR HIF1[tiab] OR MOP1[tiab] OR PASD8[tiab] OR HIF-1A[tiab] OR bHLHe78[tiab] OR HIF-1alpha[tiab] OR HIF1-ALPHA[tiab] OR "hypoxia-inducible factor 1-alpha"[tiab] OR HIF-1-alpha[tiab] OR "member of PAS protein 1"[tiab] OR "ARNT interacting protein"[tiab] OR "ARNT-interacting protein"[tiab] OR "member of PAS superfamily 1"[tiab] OR "hypoxia-inducible factor1alpha"[tiab] OR "PAS domain-containing protein 8"[tiab] OR "basic-helix-loop-helix-PAS protein MOP1"[tiab] OR "class E basic helix-loop-helix protein 78"[tiab] OR "hypoxia-inducible factor 1 alpha isoform I.3"[tiab] OR "hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor) "[tiab] OR "carbonic anhydrase 9"[tiab] OR pMW1[tiab] OR CA-IX[tiab] OR P54/58N[tiab] OR "membrane antigen MN"[tiab] OR "carbonic dehydratase"[tiab] OR "carbonate dehydratase IX"[tiab] OR "RCC-associated antigen G250"[tiab] OR "RCC-associated protein G250"[tiab] OR "renal cell carcinoma-associated antigen G250"[tiab] OR MN[tiab] OR CAIX[tiab] OR OPN[tiab] OR BNSP[tiab] OR BSPI[tiab] OR ETA-1[tiab] OR osteopontin[tiab] OR uropontin[tiab] OR nephropontin[tiab] OR"SPP1/CALPHA1 fusion"[tiab] OR "urinary stone protein"[tiab] OR "early T-lymphocyte activation 1"[tiab] OR "immunoglobulin alpha 1 heavy chain constant region fusion protein"[tiab] OR "ecreted phosphoprotein 1"[tiab]	*Determinant*
#3	#1 AND #2	*Final search*
	EMBASE	
#1	(hypox*:ab,ti OR oxygena*:ab,ti OR 'HIF':ab,ti OR HIF*:ab,ti OR OR 'hypoxia-inducible factor':ab,ti OR 'hypoxia inducible-factor':ab,ti OR 'hypoxia inducible factor':ab,ti :ab,ti 'hypoxia-inducible factor1alpha':ab,ti OR 'carbonic-anhydrase':ab,ti OR 'CA-9':ab,ti OR 'CA-IX':ab,ti OR 'carbonic dehydratase':ab,ti OR 'CAIX':ab,ti OR 'OPN':ab,ti OR 'osteopontin':ab,ti OR 'FUR':ab,ti OR 'furin':ab,ti OR 'cell hypoxia'/exp OR 'oxygenation'/exp OR 'hypoxia inducible factor'/exp)	*Domain*
#2	(('upper aerodigestive tract' OR 'UADT' OR 'Head' OR 'Neck' OR Oropharyn* OR Pharyn* OR 'Tonsil' OR 'Alveolar process' OR 'palate' OR 'oral' OR Tong* OR Laryn* OR 'mouth' OR Nasopharyn*) AND ('cance' OR neoplasm* OR carcinom* OR oncolog*) OR 'head and neck cancer'/exp)	*Determinant*
#3	#1 AND #2	*Final search*

Table S2. Critical appraisal of the excluded studies

Study	SP	SA	PF	O	C	AR	B
Bache 2006	H	L	M	L	L	M	4
Beasley 2002	H	H	M	L	M	L	4
Chan 2007	H	H	L	L	M	M	4
Chien 2012	H	L	M	L	M	M	5
Choi 2007	H	L	M	L	M	M	5
De Schutter 2005	H	L	M	L	M	L	4
Eckert 2010	H	L	L	L	M	M	4
Etiz 2013	H	H	L	L	M	M	4
Hoogsteen 2005	M	H	M	L	M	M	4
Kappler 2008	H	H	M	L	H	H	7
Klimowicz 2013	M	H	M	L	H	L	4
Kondo 2011	H	H	L	L	H	M	5
Kong 2009	M	H	L	L	M	H	4
Koukourakis 2008	H	L	L	L	H	M	5
Koukourakis 2002	H	L	M	L	M	L	4
Koukourakis 2001	H	L	L	L	M	H	5
Li 2012	H	H	M	L	H	H	7
Lin 2008	H	H	M	L	M	L	4
Liu 2008	H	H	L	L	H	H	6
Oliver 2004	H	L	M	L	H	M	6
Shou 2012	H	L	M	L	M	L	4
Uehara 2009	H	H	L	L	H	H	6

SP: study participation (Consecutive cohort? Adequately described for T-stage, N-stage and treatment?); SA: study attrition (Were all patients confirmed alive/deceased at the end of follow-up?); PF: prognostic factor (Is the PF adequately described and determined? Were cut-off values based on the literature? Was the prognostic factor determined the same for all patients?); O: Outcome (Was the outcome well defined?); C: Confounding (were confounders measured and was correction applied if appropriate?); AR: Statistical analysis and reporting (was the used statistical model adequate? Was there selective reporting of results?). B: Bias score according to QUIPS. Low = 0, Moderate = 1, High = 2 points. SA was not included.

Table S3. PRISMA checklist

Section/ topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	3
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	4
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	5
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of folL-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	4,24
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	4,24
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	27
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	24
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	5

Section/ topic	#	Checklist item	Reported on page #
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	5
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	4,18
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	5
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	5,6
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	6
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	n.a.
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a fL diagram.	5,6
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	19-23
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome-level assessment (see Item 12).	18
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group and (b) effect estimates and confidence intervals, ideally with a forest plot.	19-23, 25
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	n.a.
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	18

Section/ topic	#	Checklist item	Reported on page #
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	n.a.
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., health care providers, users, and policy makers).	8
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review level (e.g., incomplete retrieval of identified research, reporting bias).	9
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	9,10
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	10

Please note that page numbers apply to the unformatted originally submitted manuscript

Chapter 6

Poor prognosis in human papillomavirus–positive oropharyngeal squamous cell carcinomas that overexpress hypoxia inducible factor-1α

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Abstract

Background: Hypoxia induces stabilization of the transcription factor HIF-1alpha (HIF-1α), associated with (chemo-)radiotherapy resistance in oropharyngeal squamous cell carcinoma (SCC). We investigated the effect of HIF-1α expression on survival in relation to human papillomavirus (HPV) status in oropharyngeal SCC.

Methods: We conducted an immunohistochemical analysis of HIF-1α protein expression and downstream targets carbonic anhydrase-IX (CA-IX) and glucose transporter-1 (GLUT-1) in 274 patients with oropharyngeal SCC. Overall survival (OS) was analyzed in total and stratified for HPV status and treatment.

Results: In HPV-positive tumors (n = 44), HIF-1α overexpression predicted worse OS (hazard ratio [HR] = 6.23; p = .012), whereas TNM classification or treatment modality did not. In HPV-negative tumors (n = 218), advanced T and N classification and HIF-1α overexpression all independently predicted worse OS. However, the effect of HIF-1α overexpression on OS was lower in HPV-negative (HR = 1.50; p = .024) than in HPV-positive tumors.

Conclusion: HIF-1α overexpression is associated with worse OS and characterized a subgroup of patients with HPV-positive oropharyngeal SCC with poor prognosis. Possibly, patients with HIF-1α overexpressing HPV-positive tumors should not be eligible for treatment dose de-escalation

Introduction

The oropharynx is the third most commonly affected subsite in head and neck squamous cell carcinoma (SCC).[1] The 5-year overall survival (OS) rate of patients with oropharyngeal SCC remains low, around 40% to 50%. One major prognostic factor in oropharyngeal SCC is infection with a high risk of human papillomavirus (HPV).[2] HPV-associated (or HPV-positive) oropharyngeal SCC represents tumors with a distinctly different biology than "classic" smoking and alcohol-associated tumors. Tumorigenesis in HPV-positive tumors is more dependent on pathway disruptions by viral oncoproteins, whereas HPV-negative tumors are driven by genetic alterations that lead to oncogenesis.[3] Patients with HPV-positive oropharyngeal SCC are younger, have a better response to (chemo-)radiotherapy and better OS, underlining the biological differences between these two entities.[4]

Recently, tumor hypoxia has emerged as a prognostic factor in solid tumors, such as oropharyngeal SCC. Hypoxia is related to worse survival, as hypoxic tumors represent a more aggressive tumor phenotype and are more resistant to treatment with (chemo-) radiotherapy.[5] Under hypoxia, several pathways are activated to improve cellular survival and to avoid apoptosis.[6] The most important of these pathways involves the hypoxia-inducible factor 1 (HIF-1) transcription factor. This basic helix-loop-helix transcription factor consists of a HIF-1 alpha (HIF-1α) and a HIF-1 beta (HIF-1β or Aryl Hydrocarbon Receptor Nuclear Translocator [ARNT]) subunit. Both subunits are continuously synthetized, but under normoxic circumstances, the alpha subunit is quickly degraded by hydroxylation by prolyl-hydroxylases 1-3 and further ubiquitination by the Von Hippel-Lindau (VHL) protein.[7,8] As prolyl-hydroxylase activity is oxygen-dependent, hypoxia causes stabilization of HIF-1α, activating transcription of its downstream targets.[9] HIF-1α is therefore considered a hypoxia-associated protein. The targets of HIF-1α include carbonic anhydrase IX (CA-IX), which regulates intracellular pH levels and the glucose transporter-1 (GLUT-1), which increases glucose influx, required for anaerobic metabolism.[10] HIF-1α protein overexpression has been associated with worse OS and locoregional control in head and neck cancer, including oropharyngeal SCC.[11–16]

As differences in histological architecture exist between HPV-positive and HPV-negative tumors, we hypothesized that the prevalence of hypoxia in these tumors may differ as well.[17,18] In the present study, we investigated the effect of tumor hypoxia (as determined by HIF-1α protein expression) on survival in our cohort of patients with oropharyngeal SCC, mainly treated with organ-preserving treatment strategies. Moreover, we investigated the correlation between HPV-status and HIF-1α expression to find out whether tumor hypoxia may play a role in the improved survival in HPV-positive oropharyngeal SCC.

Materials and methods

Approval and consent

For this study, leftover material obtained in routine clinical care was used in an anonymized fashion. Moreover, the included patients all suffered from a disease with high morbidity and mortality. Because of both of these reasons, obtaining ethical approval or informed consent was not required, according to Dutch National Ethics Guidelines.[19]

Patient cohort

Data were collected from 274 patients presenting with a first primary oropharyngeal SCC, treated with curative intent between 1997 and 2011 at the departments of Otorhinolaryngology – Head and Neck Surgery and Oral and Maxillofacial Surgery of the University Medical Center Utrecht, The Netherlands. All patients with a first primary tumor were eligible for inclusion. The only exclusion criterion was the absence of sufficient formalin-fixed paraffin-embedded tissue in our archives for tissue-microarray (TMA) construction. HPV status was known and had been assessed as previously described.[20] Briefly, p16 immunostaining was performed, followed by a molecular HPV-detection test when positive.

Treatment strategy

All patients were discussed in our multidisciplinary head and neck team meeting, where at least one radiologist, one nuclear medicine specialist, one head and neck surgeon, one radiotherapist, one pathologist, and one medical oncologist were present. The definitive treatment strategy was ultimately decided by the patient, in accordance with his treating physicians. Most patients were treated with organ-preserving treatment (ie, primary radiotherapy [RT] or concurrent cisplatinum-based chemoradiotherapy [CRT]). Treatment of locally advanced tumors consisted mainly of surgery in earlier years. During the study period, multidisciplinary head and neck team preference for treatment of advanced disease shifted from surgery toward organ-preserving treatment through cisplatinum-based CRT. Generally, cisplatinum-based CRT was administered if tumors were functionally or technically inoperable (mostly T3–T4 tumors) and if patients were sufficiently fit to undergo this treatment. In early-stage tumors, single-modality treatment was preferred (ie, surgery or RT only). RT dose ranged from 66 to 70 Gy. Postoperative RT was administered in case of tumor-positive resection margins (R1; <1 mm), extracapsular lymph node spread, or if more than 2 intermediate risk factors were present, such as close margins (1–5 mm) or perineural, vasoinvasive, or noncohesive growth. Addition of chemotherapy to adjuvant RT was considered in the case of tumor-positive resection margins and/or extracapsular spread in lymph node metastases in patients <70 years of age and without contraindications

for chemotherapy. In our institution, contraindications for the addition of cisplatin are: (1) creatinine clearance <40 mL/min; (2) severe hearing loss; (3) active cardiovascular comorbidity; or (4) World Health Organization performance status >2.

Immunohistochemical analysis

Tissue samples of all study patients were included in a TMA, as described previously.[20] In brief, hematoxylin-eosin stained sections were reviewed by a dedicated head and neck pathologist (S.M.W.) and representative areas of tumor tissue were marked. Three 0.6-mm tumor cores per tumor were extracted and introduced in the TMA. For immunohistochemical staining, 4-micrometer slides were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked and antigen retrieval was performed by boiling in EDTA (HIF-1α, pH 9.0) or citrate buffer (CA-IX, GLUT-1, pH 6.0) for 20 minutes, rinsed with phosphate buffered saline solution, and incubated with the primary antibody (HIF-1α; BD Biosciences, Franklin Lakes, NJ; cat# 610959, lot 4073775, diluted 1:50; CA-IX; Abcam, Cambridge, UK, cat# ab15086, lot GR120038-1, 1:1000; GLUT-1; and DAKO, Glostrup, Denmark, cat# A3536, batch 117F. 1:100) for 60 minutes at room temperature (CA-IX and GLUT-1) or overnight at 4 degrees Celsius (HIF-1α). For HIF-1α, the Novolink kit (Leica, Rijswijk, The Netherlands) was used for development, in accord with the manufacturer's instructions. For CA-IX and GLUT-1, the secondary antibody used was Bright Vision (biotin free, poly-HRP anti-mouse, anti-rabbit, anti-rat antibody; Immunologic, Duiven, The Netherlands; 30 minutes), and development was performed using diaminobenzidine. Slides were subsequently counterstained with hematoxylin and coverslipped. Renal cell carcinoma tissue was used as positive and negative controls during every staining procedure. For the negative control, the primary antibody was omitted, whereas further treatment was equal.

Scoring was performed by a dedicated head and neck pathologist who was blinded to the clinical data. The percentages of nuclear (HIF-1α) or membranous (CA-IX, GLUT-1) staining tumor cells were scored. Per patient, the average of the 3 cores (or less when the cores were lost or folded) was recorded. Because each antibody and staining protocol may give other staining results, for each protein, we have identified the optimum cutoff point using the statistical methods of Williams et al.[21] This method identifies the cutoff value with the strongest correlation to OS. For HIF-1α, a cutoff of 15% or more positive staining cells was identified as the optimum cutoff point. For CA-IX and GLUT-1, the optimized cutoff points were 6% for CA-IX and 66% for GLUT-1.

Data analysis

SPSS version 22.0 (IBM, Armonk, NY) was used for all analyses. Baseline patient characteristics and biomarker expression were compared using chi-square tests for categorical variables, *t*-tests for normally distributed continuous variables, and Mann–Whitney *U* tests for non-normally distributed continuous variables. Kolmogorov–Smirnov

tests and Q-Q plots were used to assess normality. Pearson correlation analyses were performed to correlate expression of HIF-1α to expression of the downstream targets CA-IX and GLUT-1. In addition, expression of HIF-1α was compared with the clinical variables: age, sex, smoking, alcohol use, HPV status, clinical T- and N- classifications, and differentiation grade using chi-square tests, followed by a multivariate analysis using logistic regression with stepwise-backward selection using the Akaike Information Criterion ($p > .157$ for exclusion).[22]

Survival was measured from the date of the first positive biopsy until death (OS). Censored patients were confirmed alive at the time of censoring. Therefore, no patients were lost to follow-up. Survival curves were made according to the Kaplan–Meier method and compared using the log-rank test. Univariate and multivariate survival analyses were performed using the Cox proportional hazards model. The investigated variables were: age, sex, smoking, alcohol use, clinical T and N classifications, choice of treatment (surgery +/− adjuvant therapy vs primary cisplatinum-based CRT or RT), HPV status, differentiation grade, and HIF-1α, CA-IX, and GLUT-1 protein overexpression. Effect modification was investigated and stratified analyses were performed if significant interactions (ie, effect-modification) occurred. Variables that had a significant relation to OS in univariate analysis were introduced in the multivariate analysis in a stepwise-backward fashion. For internal validation, bootstrapping was performed using 5000 bootstrap samples. Statistical significance was set at $p < .05$.

Results

Patient description

Baseline patient characteristics are shown in **Table 1**. Most patients were men and the average age was 59.8 years. The majority of patients had a positive history of smoking and/or alcohol use. More than half of the patients presented with a locally advanced (T3 or T4) tumor and around two thirds of the patients had cervical lymph node metastases at the time of presentation. HPV-positivity was observed in 16.8% of cases, comparable to what was observed in a different cohort in our country.[23] Most patients were treated by primary RT, with or without cisplatin-based chemotherapy. At the end of follow-up, 152 patients (55.5%) had died. Median follow-up was 35 months for all patients (interquartile range [IQR], 15.8–67.0) and 63 months (IQR, 39.8–80) for surviving patients. HPV status could not be determined in 12 patients because of insufficient tumor tissue in biopsies for molecular testing. There were no differences in treatment between patients with HPV-positive and HPV-negative tumors.

Variable	HPV-negative n=218	HPV-positive n=44	p	Entire cohort N=274*
Age (*years*)				
Mean (SD)	59.7 (8.56)	58.2 (10.6)	NS	59.8 (9.4)
Sex				
Male	148 (67.9)	34 (77.3)	NS	190 (69.3)
Female	70 (32.1)	10 (22.7)		84 (30.7)
Smoking				
Never ever	10 (4.6)	16 (36.4)	<0.001	27 (9.9)
Quit / active smoker	206 (95.4)	28 (63.6)		245 (90.1)
Alcohol use				
Never ever	16 (7.5)	12 (27.3)	<0.001	30 (11.1)
Current or previous use	198 (95.2)	32 (72.7)		240 (88.9)
Clinical T-classification				
T_1	26 (11.9)	8 (18.6)	NS	36 (13.2)
T_2	60 (27.5)	17 (39.5)		78 (28.6)
T_3	47 (21.6)	5 (11.6)		56 (20.5)
T_{4a}/T_{4b}	85 (39.0)	13 (30.2)		103 (37.7)
Clinical N-classification				
N_0	86 (39.8)	5 (11.4)	<0.001	96 (35.3)
N_1	32 (14.8)	5 (11.4)		38 (14.0)
N_{2a}	6 (2.8)	3 (6.8)		10 (3.7)
N_{2b}	34 (15.7)	24 (54.5)		62 (22.8)
N_{2c}	46 (21.3)	6 (13.9)		53 (19.5)
N_3	12 (5.6)	1 (2.3)		13 (4.8)
HPV-status				
Positive	-	-	-	44 (16.8)
Negative	-	-		218 (83.2)
Localization				
Tonsillar area	47 (21.6)	11 (25.0)	NS	109 (39.8)
Base of tongue/vallecula	80 (36.7)	22 (50.0)		58 (21.2)
Other	91 (41.7)	11 (25.0)		107 (39.1)
Differentiation				
Well	4 (2.4)	0 (0)	0.040	4 (2.0)
Moderate	125 (74.9)	15 (53.6)		146 (71.6)
Poorly	37 (22.2)	12 (42.9)		52 (25.5)
Undifferentiated	1 (0.6)	1 (3.6)		2 (1.0)
Treatment				
Surgery only	14 (6.4)	1 (2.3)	NS	15 (5.5)
Surgery + PORT	57 (26.1)	9 (20.5)		69 (25.2)
Surgery + POCRT	4 (1.8)	1 (2.3)		5 (1.8)
Primary RT	56 (25.7)	13 (29.5)		74 (27.0)
Primary CRT	87 (39.9)	20 (45.5)		111 (40.5)
HIF-1a				
Positive (≥ 15%)	87 (42.0)	23 (56.1)	NS	146 (56.2)
Negative (< 15%)	120 (58.0)	19 (43.9)		114 (43.8)
CA-IX				
Positive (≥6%)	73 (34.6)	21 (47.7)	NS	99 (37.1)
Negative (6%)	138 (65.4)	23 (52.3)		168 (62.9)
Glut-1				
Positive (≥66%)	59 (29.2)	5 (12.5)	0.029	65 (25.7)

Variable	HPV-negative n=218	HPV-positive n=44	p	Entire cohort N=274*
Negative (<66%)	143 (70.8)	35 (87.5)		188 (74.3)
Follow-up all patients				
Median months (IQR)	32.0 (14.0 – 65.0)	51.5 (29.5 – 75.0)	-	35.0 (15.8 – 67.0)
Follow-up surviving patients				
Median months (IQR)	57.0 (37.5 – 78.5)	66.0 (46.3 – 81.3)	-	63.0 (39.8 – 80.0)
Deceased at end of follow-up	137 (62.8)	10 (22.7)	-	152 (55.5)

Table 1 | Baseline patient characteristics by HPV-status. All values are shown as n (%) unless stated otherwise. Abbreviations: HPV: human papillomavirus, IQR: interquartile range, PORT: postoperative radiotherapy, POCRT: postoperative chemoradiation. RT: radiotherapy. CRT: chemoradiotherapy. Categorical variables are displayed as number (percentage) of patients in whom these data were available. Some baseline characteristics were not available and were scored as missing. * Total number of patients was 274, HPV-status could not be determined in 12 patients.

Hypoxia-inducible factor-1-alpha is frequently overexpressed and correlated to carbonic anhydrase-IX and glucose transporter-1 overexpression

HIF-1α protein expression was observed as a nuclear staining pattern (**Figure 1A and B**). CA-IX and GLUT-1 protein expression were observed as a membranous staining pattern (**C and D**). HIF-1α positivity was observed in slightly more than half of the cases (146/260; 56.2%). CA-IX staining was positive in 37.1% (99/267) and GLUT-1 staining was positive in 25.7% of patients (65/253). HIF-1α expression was significantly associated with increased CA-IX expression (r = 0.193; p = .002), as well as GLUT-1 expression (r = 0.204; p = .001) as shown in **Table 2**.

No difference in hypoxia-inducible factor-1-alpha expression between human papillomavirus-positive and human papillomavirus-negative tumors

Correlations between HIF-1α overexpression and clinical variables are shown in **Supplementary Table S1**, online only. HIF-1α overexpression was observed more often in men than in women (p = .027) and in patients with locally advanced or T3/T4 tumors (p = .034). Other clinical characteristics did not correlate to HIF-1α protein expression. In particular, we did not observe a difference in HIF-1α positivity between HPV-positive and HPV-negative tumors (43.9% vs 58.0%; p = .098). A multivariate analysis with age, sex, T and N classifications, differentiation grade, and HPV status revealed that male sex and advanced T classification were independently associated with HIF-1α positivity. HPV-positivity correlated with lower alcohol use, but no correlation was observed with smoking history. In addition, HPV-positive tumors had lower T and higher N classifications, as has been previously described.[24] A positive smoking history correlated with a history of alcohol use.

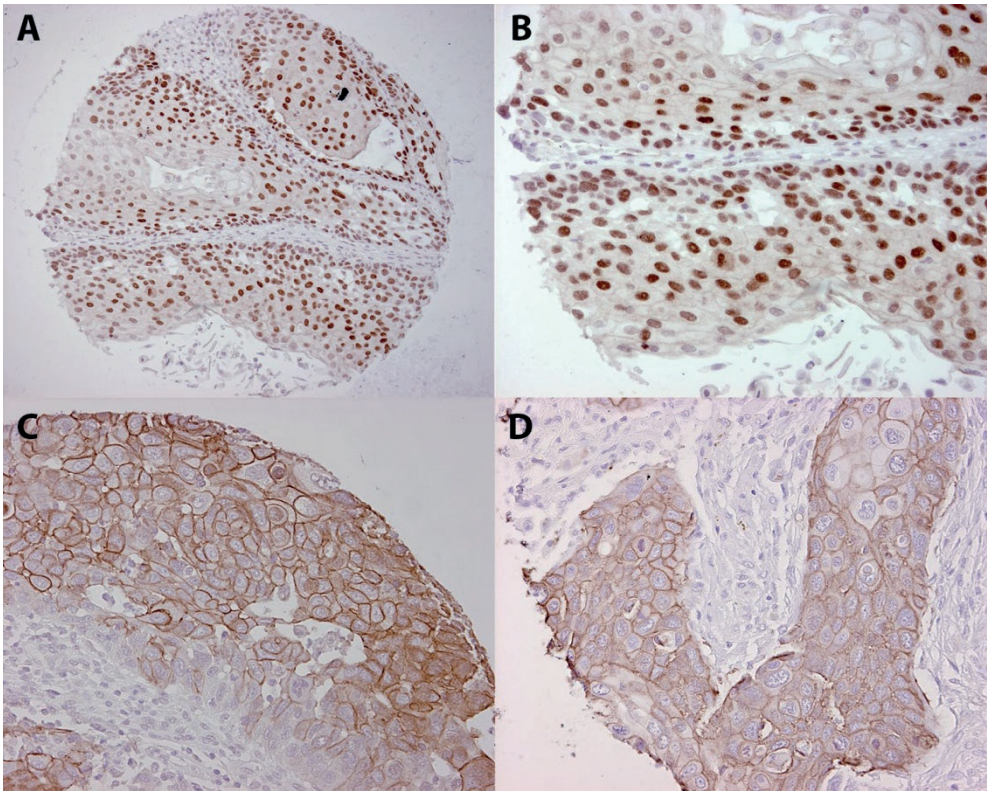

Figure 1 | Staining patterns of HIF-1a, CA-IX and GLUT-1. Example of a 0.6mm tissue microarray core with positive nuclear staining for HIF-1a (A, magnification 10x; B, magnification 20x). A membranous staining pattern was observed for CA-IX (C) and GLUT-1 (D).

		HIF-1a expression	CA-IX expression
CA-IX expression	*r*	0.193	
	p	0.002	
GLUT-1 expression	*r*	0.204	0.189
	p	0.001	0.003

Table 2 | Correlation of HIF-1a expression to downstream targets. Significant correlations are shown in bold. HIF-1a positivity was significantly associated with higher expression of CA-IX and GLUT-1. Abbreviations: *r*: Pearson correlation coefficient

Overexpression of hypoxia-inducible factor-1-alpha, but not carbonic anhydrase-IX and glucose transporter-1, is associated with worse overall survival, independent of human papillomavirus status

On univariate analysis, we investigated the association to OS for the patient and tumor characteristics described above. Age (p = .045), smoking (p =.023), clinical T classification (p < .001), clinical N classification (p = .006), choice of treatment (p = .035, in favor of surgery), overexpression of HIF-1α (p = .007; **Figure 2**) and HPV-status (p < .001) were significantly associated with OS in the entire cohort (**Table 3**). CA-IX expression had no association to OS. GLUT-1 positivity was significantly associated to OS (p = .026). In the multivariate analyses, age, clinical T and N classification, choice of treatment, HIF-1α expression, and HPV status retained their significance.

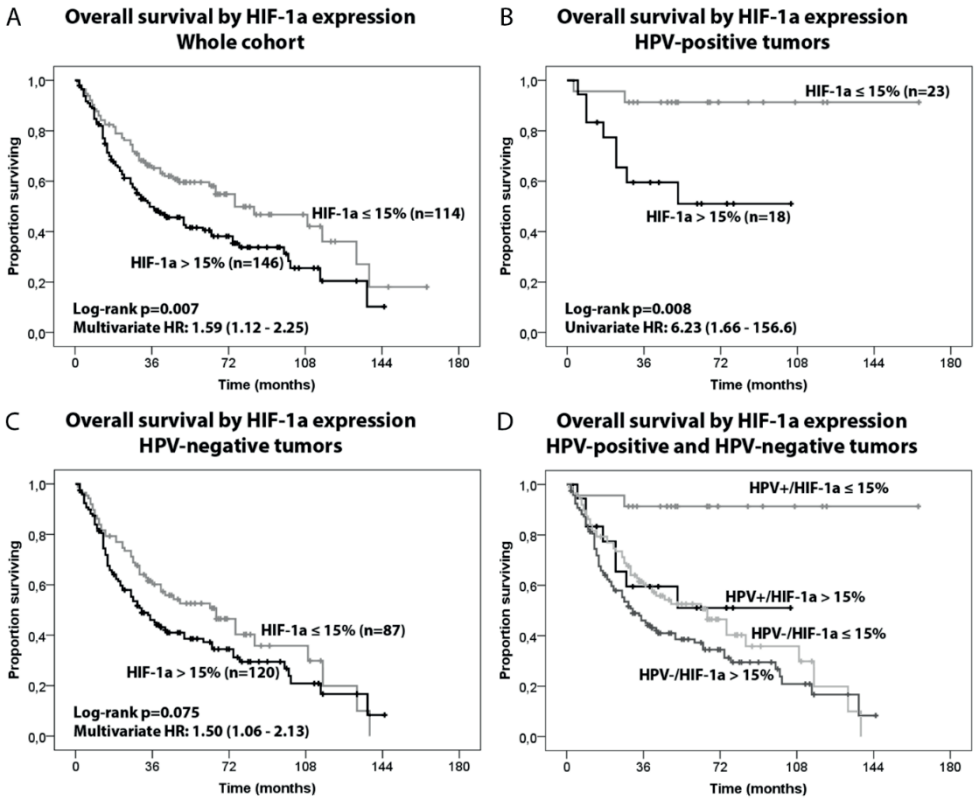

Figure 2 | Survival curves. Kaplan-Meier curves stratified according to HIF-1a expression above 15% nuclear expression for all patients (a), and those with HPV-negative (b) or HPV-positive tumors (c). The overlay of figures 2b and 2c (d) shows that the prognosis of HPV-positive/HIF-1a positive patients is comparable to HPV-negative patients.

	Univariate analysis			Multivariate analysis		
Characteristic	HR	95%-CI	p	HR	95%-CI	p
Age						
Per year increase	1.019	1.000 – 1.039	**.045**	1.025	1.004 – 1.047	**0.019**
Sex						
Male vs. female	0.811	0.565 – 1.166	.259	-	-	-
Smoking						
Current/quit vs. never ever	2.294	1.124 – 4.684	**.023**	NS	NS	NS
Alcohol						
Current/quit vs. never ever	1.386	0.784 – 2.450	0.261	-	-	-
clinical T-classification						
cT3-4 vs. cT1-2	2.067	1.457 – 2.933	**<.001**	1.849	1.276 – 2.679	**0.001**
clinical N-classification						
cN+ vs. cN0	1.644	1.154 – 2.342	**.006**	2.424	1.629 – 3.608	**<.001**
Treatment						
CC(RT) vs. surgery*	1.469	1.028 – 2.100	**0.035**	1.518	1.041 – 2.213	**0.030**
HPV						
Negative vs. positive	3.536	1.859 – 6.725	**<.001**	3.889	2.018 – 7.498	**<.001**
Differentiation grade						
I-II vs. III-IV	0.716	0.460 – 1.115	.139	-	-	-
HIF-1a expression						
Negative vs. positive	1.588	1.132 – 2.229	**.007**	1.686	1.184 – 2.401	**0.004**
CA-IX expression						
Below 6 vs. above 6%	1.332	0.957 – 1.854	.089	-	-	-
GLUT-1 expression						
Below 66 vs. above 66%	1.502	1.049 – 2.151	**.026**	NS	NS	NS

Table 3 | Univariate and multivariate analyses of Overall Survival. Univariate and multivariate Cox-regression analysis for overall survival. Significant values are shown in bold. Values significant on univariate analysis are introduced into the multivariate model through a backward stepwise fashion. Smoking and GLUT-1 expression above 66% were not associated with survival in the multivariate analysis. Abbreviations: HR: hazard ratio, CI: confidence interval, NS: not significant, - indicates variable was not included in multivariate analysis, CC(RT): radiotherapy with or without adjuvant chemotherapy, HPV: human papillomavirus. *Surgery with or without adjuvant treatment

Effect of hypoxia-inducible factor-1-alpha on overall survival is significantly stronger in human papillomavirus-positive than in human papillomavirus-negative tumors

HPV status proved to be a significant effect modifier in the association between HIF-1α expression and OS, meaning that the prognostic value of HIF-1α overexpression for OS was significantly different in patients with HPV-positive tumors than in those with HPV-negative tumors (p = .036 for the interaction term). Therefore, we performed analyses stratified by HPV status (**Table 4**; **Figure 2**).

In patients who were HPV-positive (n = 44), HIF-1α overexpression (p = .012) and age (p = .001) were significantly associated with survival. As only 10 deaths occurred in this subgroup, multivariate analysis was not possible. The effect of T and N classifications on OS did not reach statistical significance (p = .093 and p = .902, respectively), although there was a tendency toward worse survival in patients with higher T- or N-classifications (Supplementary Figure S1, online only). Smoking (p = .624) or treatment strategy did not have a significant association to OS. In the larger group of patients who had an HPV-negative tumor (n = 218) HPV-negative (n = 218), T-classification, N-classification, and HIF-1α expression were all independently associated with OS, although the hazard ratio (HR) was much smaller for HIF-1α in this subgroup compared with patients who were HPV-positive (HR = 1.50 in HPV-negative disease vs HR = 6.23 in HPV-positive disease).

Hypoxia-inducible factor-1-alpha and carbonic anhydrase-IX are independently associated to overall survival in cisplatinum-based chemoradiotherapy/radiotherapy treated patients

Subgroup analyses were performed for patients who received primary surgery (n = 89) and those who underwent cisplatinum-based CRT/RT (n = 185). An in-depth description of the analyses of these subgroups is provided in Supplementary Tables S2–S4, online only). In the surgery-treated subgroup, 43 patients died during follow-up. In a multivariate Cox regression analysis, only higher clinical N classification was associated with worse OS. There was no association between HIF-1α expression and OS. In the (chemo-) radiotherapy treated patients, age (p = .004), advanced T-classification, and N-classification (both p = .001), HIF-1α overexpression (p = .023), CA-IX overexpression (p = .002), and negative HPV status (p = .001) were all independently related to worse OS. The addition of cisplatin versus RT alone did not result in significantly better survival.

		HR	95% CI	p
HPV-positive patients (n=44)*				
HIF-1a expression	>15% vs. <15%	6.23	1.66 – 156.64	0.012
Age	Per year increase	1.09	1.03 – 1.22	0.001
HPV-negative patients (n=218)**				
T-classification	T3-4 vs. T1-2	1.80	1.26 – 2.73	0.001
N-classification	N+ vs. N0	2.42	1.63 – 3.75	<.001
HIF-1a expression	≥15% vs. <15%	1.50	1.06 – 2.13	0.024

Table 4 | Overall survival analyses stratified for HPV-status. Because of effect-modication by HPV-status, stratified analysis were performed for HPV-positive and HPV-negative patients. In HPV-positive patients, HIF-1a overexpression and age, were univariate significant predictors of overall survival. Interestingly, the effect of clinical T- and N-classification on survival did not reach statistical significance in this subgroup. In HPV-negative patients, age, smoking and type of therapy were not significantly associated with overall survival. Bootstrapping was performed for the final multivariate model (HPV-negative) and for each

univariate variable in HPV-positive patients. Abbreviations: HR: hazard ratio, CI: confidence interval. *Univariate bootstrapped Cox-regression analysis. **Multivariate stepwise backward Cox-regression analysis, included variables: age, smoking, clinical T-classification, clinical N-classification, type of therapy and HIF-1a expression. Bootstrapping was performed on the final model.

Discussion

In this cohort of 274 patients with oropharyngeal SCC treated mainly with cisplatinum-based CRT and RT, we investigated the prognostic value of the hypoxia-associated protein, HIF-1α, and its downstream targets CA-IX and GLUT-1. We found that HIF-1α overexpression was a predictor of worse OS, independent of HPV status, T-classification, and N-classification. CA-IX and GLUT-1 expression did not predict treatment outcome independent of HIF-1α in the entire cohort, although CA-IX overexpression was independently related to worse OS in cisplatinum-based CRT/RT-treated patients. Although HIF-1α overexpression was significantly associated with decreased survival in both HPV-positive and HPV-negative tumors, HPV status proved to be an effect modifier: the effect of HIF-1α overexpression on survival was significantly stronger in HPV-positive tumors. Moreover, T- and N-classifications were significant predictors for survival in HPV-negative, but not in HPV-positive tumors.

In the current era of cancer treatment, we aim for personalized, patient-tailored treatment. At this moment, clinical decisions are based only on the disease extent established through TNM classifications. However, current research focuses on the molecular tumor biology to identify biomarkers to predict prognosis and to help select the optimal treatment for each patient. These biomarkers should have a decisive influence on the choice of treatment in the near future. Models as presented in the work of Hanahan and Weinberg describe cancer as part of a microenvironment, with various hallmarks that may pose as therapeutic targets in the future.[25] One promising biomarker within the tumor microenvironment is hypoxia, which may develop because of rapid tumor outgrowth and decreases sensitivity to treatment.[5,15]

Under hypoxia, the transcription factor HIF-1 is stabilized and activated, as degradation of the HIF-1α subunit is an oxygen-dependent process. Thus, HIF-1α is considered a biomarker of hypoxia.[9] Hypoxia is more often present in larger tumors, as shown by higher HIF-1α overexpression in tumors with advanced T-classifications in the present and previous studies.[13,15] Hypoxia leads to a reduced response to radiation, as oxygen has an important role in inducing DNA damage and cell death during RT.[26,27] This was confirmed in our cohort, in which patients with HIF-1α overexpressing tumors had worse OS compared with tumors without overexpression of HIF-1α. This finding is in line with 2 smaller studies on patients with oropharyngeal SCC treated with RT only, which did not take HPV-status into account.[11,14] Moreover, in 2013, Rahimi *et al* found a significant association between HIF-1α

overexpression and survival on univariate analysis of 106 patients with oropharyngeal SCC.[12] However, in their multivariate survival analysis that included p16 expression as a surrogate marker for HPV-positivity, HIF-1α overexpression did not retain its significance. This may have been due to a lack of power. In addition, effect modification by HPV status, as we describe in the present study, was not investigated and may have influenced their results.

HPV status has emerged as an important predictor of treatment sensitivity and outcome in oropharyngeal SCC. Moreover, HPV-positive tumors have been shown to represent a different tumor biology, in which tumorigenesis is driven by viral oncoproteins.[3] HPV-positive tumors present with different histological features than HPV-negative tumors.[17,18] Differences between HPV-positive and HPV-negative tumors were also observed in diffusion-weighted MRI studies.[28,29] We hypothesized that the prevalence of hypoxia may differ between HPV-positive and HPV-negative tumors. This hypothesis could not be confirmed, as we did not observe a difference in HIF-1α expression between HPV-positive and HPV-negative tumors.

We also hypothesized that HPV status would influence the relation between hypoxia and therapy response. Indeed, we found that survival of patients who were HPV-positive with no or low expression of HIF-1α was excellent. In contrast, patients with HPV-positive HIF-1α overexpressing tumors had a prognosis that was comparable to HPV-negative tumors. In HPV-negative tumors, the OS was low and was influenced by HIF-1α overexpression with a lower effect size (HR). Surprisingly, similar results were not obtained in a previous study on 230 patients with oropharyngeal SCC (113 patients were HPV-positive).[13] In this cohort, HIF-1α overexpression was not significantly associated with survival in the entire cohort or when stratified by HPV status. It is possible that a different staining protocol and cutoff for positivity may explain this difference, even though Hong *et al* used the same antibody clone as was used in our study. Another explanation may be that the majority of patients in the cohort of Hong *et al* had received primary surgery with or without adjuvant treatment: 56% compared to 33% in our cohort. It is possible that excision of the tumor removes the hypoxic fraction, increasing oxygenation and the susceptibility to the adjuvant therapy.[30] Unfortunately, no separate analyses were performed for surgically treated and non-surgically treated patients in this study.

Hong *et al* also describe a correlation between HIF-1α overexpression and tumor grade.[13] Although this was observed in our study, this correlation did not reach statistical significance. The treatment strategy may also play a role in this, because in patients treated with RT only, tissue from incisional biopsies is available. It was recently described that histological features of incisional biopsies correspond poorly to the resected whole tumor.[31] This may have also played a role in not finding a significant correlation between HIF-1α overexpression and differentiation grade.

In this study, we observed that HIF-1α was associated with survival in cisplatinum-based CRT/RT-treated patients, but not in patients treated with surgery. This finding supports the hypothesis that hypoxia leads to reduced sensitivity to RT or CRT and is clinically less relevant when a surgical approach is used. However, it should be taken into account that the subgroup of surgically treated patients in our cohort was small and the lack of a significant outcome may have been due to a lack of power. Still, surgery followed by adjuvant therapy on indication may be a preferable treatment option for hypoxic tumors. This hypothesis should be further explored in future studies.

Even though HIF-1α regulates transcription of CA-IX, we found only a weak correlation between HIF-1α and CA-IX overexpression. In addition, we observed that CA-IX overexpression was associated with worse OS, independent of HIF-1α overexpression in cisplatinum-based CRT/RT-treated patients. Expression of CA-IX is regulated by HIF-1α through hypoxia-responsive elements located directly upstream of the promotor region of the gene coding for CA-IX. However, CA-IX expression is also regulated through the mitogen-activated protein kinase or phosphotidylinositol-3-kinase pathways, which may also infer resistance to RT independent of hypoxia.[10,32] Therefore, CA-IX overexpression may also predict worse OS in cisplatinum-based CRT/RT-treated patients, independent of HIF-1α.

Our findings are important for clinical practice, as treatment deintensification trials are currently undertaken for patients with HPV-positive tumors.[33] We observed that HIF-1α overexpression characterized a subgroup of HPV-positive tumors that have a comparable outcome to patients with HPV-negative tumors. Therefore, treatment deintensification may not be appropriate for this subgroup.

Several limitations of this study should be addressed. The first is the relatively low number of patients who were HPV-positive, in line with the low prevalence of HPV-positive oropharyngeal SCC in The Netherlands of around 19%.[34] Because of this, we could only perform univariate, but not multivariate survival analyses in the relatively small subgroup of patients who were HPV-positive. Moreover, we found that in patients with HPV-positive oropharyngeal SCC, HIF-1α overexpression had a significant relation with OS, whereas the effect of higher T- and N-classifications on OS did not reach statistical significance. This was probably because of the relatively low sample size, as we did find that patients with higher T- or N-classifications tend to have worse survival. Ang *et al* have already described that N classification (N0–N2a vs N2b–N3) can stratify patients with HPV-positive oropharyngeal SCC in groups with low-risk and moderate-risk of death.[2] Likely, the effect size of the relation between OS and T and N classification was not sufficient to be detected as a statistically significant difference in our relatively small group of patients with HPV-positive oropharyngeal SCC. Still, it is interesting that HIF-1α overexpression did have a significant association with OS in HPV-positive oropharyngeal SCC, despite the sample size. Therefore, our findings should be validated in multivariate analysis in a larger and independent cohort

of patients with HPV-positive oropharyngeal SCC to confirm whether HIF-1α overexpression indeed characterizes a group of HPV-positive oropharyngeal SCC associated with poor survival. Such a study should include stratification for HPV status and treatment.

Second, we established optimal cutoff points using the statistical methods of Williams et al.[21] This method identifies the cutoff point with the strongest potential to differentiate between binary or survival outcomes. In this method, there is a risk of overfitting the data. To control for the risk of overfitting, we performed internal validation using bootstrapping, a method that is preferable to splitting data in a training and validation set.[35] In bootstrapping analyses, random samples are drawn from the dataset to correct for overfitting. Still, external validation in an independent cohort would be preferable in the future to confirm both the cutoff value and the prognostic value of HIF-1α expression in patients with HPV-positive oropharyngeal SCC.[36]

Conclusion and recommendation

HIF-1α protein overexpression is significantly associated to worse OS in patients with oropharyngeal SCC. In our cohort, this association is particularly strong in patients with HPV-positive tumors. Future research should confirm this finding and elucidate the mechanism of the observed differences in hypoxia-related treatment resistance between HPV-positive and HPV-negative tumors and how these findings could be translated into clinical practice. Better understanding of the clinical relevance of hypoxia may improve treatment selection and may ultimately contribute to better personalized cancer care.

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		Sex		Alcohol		Smoking		HPV		cT-stage		cN-stage		Differentiation Grade	
		M	F	Never	Q/A	Never	Q/A	Neg	Pos	T1/2	T3/4	N0	N+	I/II	III/IV
HIF-1a	Negative	**38.4**	**55.4**	53.3	42.0	53.8	42.7	42.0	56.1	**51.9**	**38.6**	42.0	45.0	40.3	50.0
	Positive	**61.6**	**44.6**	46.7	58.0	46.2	57.3	58.0	43.9	**48.1**	**61.4**	58.0	55.0	59.7	50.0
	p	**0.010**		NS		NS		NS		**0.034**		NS		NS	
Sex	Male			56.7	71.7	66.7	69.8	67.9	77.3	67.0	70.8	67.7	69.9	74.0	66.7
	Female			43.3	28.3	33.3	30.2	32.1	22.7	33.0	29.2	32.3	30.1	26.0	33.3
	p			NS		NS		NS		NS		NS		NS	
Alcohol	Never					**48.1**	**7.0**	**7.5**	**27.3**	8.3	12.5	**5.3**	**14.4**	11.6	11.1
	Quit/Active					**51.9**	**93.0**	**92.5**	**72.4**	91.7	87.5	**94.7**	**85.6**	88.4	88.9
	p					**<0.001**		**<0.001**		NS		**0.025**		NS	
Smoking	Never							**4.6**	**36.4**	11.7	8.1	6.3	12.1	8.1	16.7
	Quit/Active							**95.4**	**63.6**	88.3	91.9	93.8	87.9	91.9	83.3
	p							**<0.001**		NS		NS		NS	
HPV	Negative									**78.0**	**87.5**	**94.5**	**76.9**	**89.6**	**74.5**
	Positive									**22.0**	**12.5**	**5.5**	**23.1**	**10.4**	**25.5**
	p									**0.041**		**<0.001**		**0.008**	
cT-classification	T1/T2											45.8	37.7	**35.3**	**53.7**
	T3/T4											54.2	62.3	**64.7**	**46.3**
	p											NS		**0.018**	
cN-classification	N0													36.2	29.6
	N+													63.8	70.4
	p													NS	

Table S1 | Correlation of HIF-1a status to clinical characteristics. For HIF-1a, the cut-off of 15% nuclear expression was used. Values are column percentages. Statistical significance was tested using Chi-squared tests.

	Surgery +/- adjuvant N=89	CCRT / RT N=185	*p*
Age (years)			
Mean (SD)	60.42 (9.12)	58.76 (8.78)	0.155
Sex			
Male	62 (69.7)	128 (69.2)	0.937
Female	27 (30.3)	57 (30.8)	
Smoking			
Never ever	9 (10.3)	18 (9.7)	0.874
Quit / active smoker	78 (89.7)	167 (90.3)	
Alcohol use			
Never ever	9 (10.6)	21 (11.4)	0.853
Current or previous use	76 (89.4)	164 (88.6)	
cT-classification			
T_1	22 (24.7)	14 (7.6)	**<0.001**
T_2	29 (32.6)	49 (26.6)	
T_3	19 (21.3)	37 (20.1)	
$T_{4a/4b}$	19 (21.3)	84 (45.7)	
cN-classification			
N_0	35 (40.2)	61 (33.0)	**0.004**
N_1	16 (18.4)	22 (11.9)	
N_{2a}	7 (8.0)	3 (1.6)	
N_{2b}	18 (20.7)	44 (23.8)	
N_{2c}	10 (11.5)	43 (23.2)	
N_3	1 (1.1)	12 (6.5)	
HPV-status			
Positive	11 (12.8)	33 (18.8)	0.226
Negative	75 (87.2)	143 (81.3)	
Localization			
Tonsillar area	44 (49.4)	65 (35.1)	0.047
Tongue base/vallecula	13 (14.6)	45 (24.3)	
Other	32 (36.0)	75 (40.5)	
Differentiation			
Well	1 (1.4)	3 (2.3)	0.624
Moderate	55 (75.3)	91 (69.5)	
Poorly	17 (23.3)	35 (26.7)	
Undifferentiated	0 (0.0)	2 (1.5)	
Treatment			
Surgery only	15 (16.9)	-	NA
Surgery + PORT	69 (77.5)	-	
Surgery + POCRT	5 (5.6)	-	
Primary RT	-	74 (40.0)	
Primary CRT	-	111 (60.0)	
HIF-1a			
Positive (≥ 15%)	51 (60.0)	95 (54.3)	0.384
Negative (< 15%)	34 (40.0)	80 (45.7)	
CA-IX			
Positive (≥ 10%)	28 (32.7)	56 (31.1)	0.860
Negative (<10%)	59 (67.8)	124 (68.9)	
Glut-1			
Positive (≥ 10%)	67 (80.7)	149 (87.6)	0.143

	Surgery +/- adjuvant N=89	CCRT / RT N=185	*p*
Negative (<10%)	16 (19.3)	21 (12.4)	
Deaths	43 (48.3)	109 (58.9)	

Table S2 | Baseline patient data, stratified by treatment strategy. CCRT: concurrent chemoradiation, RT: radiotherapy, PORT: post-operative radiotherapy, POCRT: post-operative chemoradiation, NA: not applicable. Patients with the data available are shown.

	Surgery +/- adjuvant therapy			CCRT/RT		
Characteristic	HR	95%-CI	p	HR	95%-CI	p
Age						
Per year increase	0.992	0.956 – 1.029	0.667	1.034	1.011 – 1.057	0.003
Sex						
Male vs. female	0.657	0.321 – 1.345	0.251	0.895	0.587 – 1.365	0.608
Smoking						
Current/quit vs. never ever	0.040	0.001 – 2.501	0.127	0.642	0.312 – 1.322	0.229
Alcohol						
Current/quit vs. never ever	2.116	0.507 – 8.820	0.304	1.275	0.682 – 2.381	0.447
cT-classification						
cT3-4vs. cT1-2	1.173	0.635 – 2.166	0.610	2.630	1.667 – 4.150	<0.001
cN-classification						
cN+ vs. cN0	1.929	0.968 – 3.842	0.062	1.494	0.986 – 2.263	0.058
Treatment						
CCRT versus RT	*Reference category*			1.299	1.060 – 1.592	0.012
HPV						
Negative vs. positive	27.25	0.803 – 925.4	0.066	2.759	1.436 – 5.301	<0.001
Differentiation grade						
I-II vs. III-IV	0.569	0.220 – 1.472	0.245	0.730	0.441 – 1.208	0.221
HIF-1a expression						
Positive vs. negative	1.665	0.842 – 3.292	0.143	1.626	1.096 – 2.415	0.016
CA-IX expression						
Below 10 vs. above 10%	0.773	0.391 – 1.527	0.458	1.527	1.020 – 2.285	0.040
GLUT-1 expression						
Below 10 vs. above 10%	1.359	0.528 – 3.498	0.525	1.222	0.666 – 2.241	0.517

Table S3 | Univariate survival analyses stratified by treatment strategy. In surgery-treated patients, none of the clinicopathological variables had a significant relation to OS in the univariate analyses, possibly due to lack of power in this small subgroup.

	Surgery +/- adjuvant therapy			CCRT/RT		
Characteristic	HR	95%-CI	p	HR	95%-CI	p
Age						
Per year increase		NA		1.036	1.012 – 1.062	0.004
cT-classification						
cT3-4vs. cT1-2		NS		2.332	1.435 – 3.791	0.001
cN-classification						
cN+ vs. cN0	2.496	1.239 – 5.027	0.010	2.316	1.419 – 3.781	0.001
Treatment						
CCRT versus RT		*Reference category*			NS	
HPV						
Positive vs. negative		NS		3.311	1.677 – 6.535	0.001
HIF-1a expression						
Positive vs. negative		NS		1.687	1.109 – 2.566	0.014
CA-IX expression						
Below 10 vs. above 10%		NA		1.569	1.020 – 2.413	0.040

Table S4 | Multivariate survival analyses stratified by treatment strategy. NA: not applicable, these variables were not included in the multivariate analysis. NS: not significant. In surgery-treated patients, higher N-stages resulted in significantly worse OS, when corrected for clinical T-stage, HPV-status and HIF-1a overexpression. In CCRT/RT-treated patients, all investigated variables, with exemption of treatment strategy, were independently associated to OS.

Chapter 7

HIF-1a expression and differential effects on survival in patients with oral cavity, larynx, and oropharynx squamous cell carcinomas

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Abstract

Background: Hypoxia is a negative prognostic factor in head and neck squamous cell carcinomas. Under hypoxia, the hypoxia-inducible factor (HIF)-1a transcription factor is overexpressed. We investigated whether there were site differences in HIF-1a expression and its effect on patient outcomes per subsite.

Design/Method: A total of 941 patients with HNSCC in the squamous cell carcinoma of the oropharynx (OPSCC, n = 302), oral cavity (OSCC, n = 391), or larynx (LSCC, n = 248) were included. Expression of HIF-1a in tissue samples was investigated using immunohistochemistry. Overall survival (OS), disease-free survival (DFS), and locoregional control (LRC) were analyzed.

Results: HIF-1a expression was higher in OSCC than in LSCC and OPSCC. High HIF-1a expression led to worse prognosis in OPSCC (OS p = .029, DFS p = .085) and LSCC (OS p = .041, DFS p = .011) and better prognosis in OSCC (OS p = .055, DFS p = .012). There was no association between HIF-1a and LRC.

Conclusions: High HIF-1a expression is related to poor outcome in OPSCC and LSCC and better outcome in OSCC.

Introduction

Hypoxia is a state of a reduced tissue oxygen tension and is often observed within the tumor microenvironment. Processes in a tumor that cause hypoxic cells to proliferate in these circumstances are also the basis for a more aggressive phenotype and a higher likelihood of metastasis.[1,2] The hypoxia-inducible factor 1 (HIF-1) pathway has an important role in cellular survival under hypoxia. In head and neck squamous cell carcinoma (HNSCC), high expression of HIF-1alpha (HIF-1a) has been shown to lead to worse prognosis.[3]

HNSCC comprises carcinomas arising in mucosal lining of the upper aero-digestive tract. Biologically, HNSCCs were considered a homogenous group of tumors arising in different anatomic regions, or sites, within the upper aero-digestive tract. Pathogenesis of HNSCC is generally attributed to accumulation of mutations in oncogenes and tumor-suppressor genes because of DNA damage that can be caused by smoking and drinking.[4] However, HPV-related oropharyngeal tumors have been identified as a distinct subgroup within HNSCC. In these tumors, HPV onco-proteins (especially E6 and E7) cause changes in signaling pathways that lead to carcinogenesis, rather than accumulation of mutations. Clinically, HPV-positive oropharyngeal tumors show better response to radiotherapy treatment in contrast to HPV-negative oropharyngeal tumors.[5] Interestingly, survival benefits for HPV-related tumors were not identified in non-oropharyngeal sites.[6,7] These findings highlight the genetic and biological heterogeneity of HNSCC.

These biological differences between HNSCCs of different sites warrant further investigation into HIF-1a expression and its effect on clinical outcome. The aim of the present study was to compare both the amount of HIF-1a expression and the effect of HIF-1a overexpression on clinical outcome between sites. We studied the effect of HIF-1a expression in four well-documented patient cohorts, all treated in our center, that encompass the three major sites of HNSCC: the oral cavity, oropharynx, and larynx. Because all patient tissues were stained using an identical staining protocol, we were able to compare the amount and effect of HIF-1a expression across different sites.

Materials and methods

Patient cohort

Patients diagnosed with a first primary oral, oropharyngeal, or laryngeal carcinoma and treated with curative intent at the University Medical Center Utrecht in the Netherlands were included in this retrospective study. All data and tissue samples were handled according to the General Data Protection Regulation (GDPR).

Oral cancer cohorts

Two previously described patient cohorts were analyzed in the present study.[8,9] All 391 patients had been diagnosed with an OSCC between 1996 and 2010. OSCC patients treated with primary (chemo)radiotherapy (CRT) were not included in this study, because generally these patients had (functionally) irresectable disease, or either did not wish or were not fit to undergo extensive surgery.

Oropharyngeal cancer cohort

The oropharyngeal cancer (OPSCC) cohort has also been previously described.[10] A total of 274 patients were included who were diagnosed with an OPSCC between 1997 and 2011 and had tumor tissue available in the pathology archives for tissue microarray (TMA) construction. In addition, 28 patients with an OPSCC included in the study by Van Hooff *et al* were also included.[8]

Laryngeal cancer cohort

The laryngeal cancer (LSCC) cohort consisted of all consecutive patients diagnosed with a laryngeal squamous cell carcinoma between January 2007 and June 2014. Exclusion criteria were (a) no tissue available at the University Medical Center Utrecht, (b) insufficient material for TMA construction, and (c) receiving systemic therapy for another, non HNSCC, malignancy within less than 5 years before diagnosis. Given that this was a retrospective cohort study, there were no means to collect additional tissue in case of insufficient material. A total of 248 patients were included.

Treatment strategy

For all patients, a treatment proposition was offered based on the clinical TNM-staging (seventh edition), the most recent guidelines during the time of treatment and after discussion in the weekly head and neck oncology multidisciplinary team (MDT) meeting. Adjuvant treatment was advised based on the criteria in **Appendix S1**. In our center, this team consists of at least an otolaryngologist, maxillofacial surgeon, radiation oncologist, medical oncologist, radiologist, nuclear physician, and pathologist, all specialized in treatment of HNSCC. The final treatment was ultimately decided by the patient in concurrence with his or her treating physicians.

Definitions

HPV-status was determined using the algorithm described by Smeets *et al*: a p16 staining was performed, followed by an HPV-PCR if positive.[11] HPV-positivity outside the oropharyngeal site was rare and was only deemed clinically relevant in patients with oropharyngeal cancer. HPV-status was therefore categorized as “positive,” “negative,” or “non-oropharyngeal site”.[8,12] Patients treated with radiotherapy and cisplatin or

carboplatin, as well as patients treated with radiotherapy and cetuximab, were analyzed as a single treatment group (chemo-radiotherapy). All tumors were staged according to the UICC TNM seventh edition.

Immunohistochemistry

All tissues were leftover materials following routine clinical care. All clinical patient data were analyzed anonymously. Therefore, obtaining informed consent or ethical board approval was not necessary according to the Dutch best practice guidelines at the time of the study (www.federa.org).

Immunohistochemistry was performed on TMAs, which is widely accepted to investigate protein expression in large cohorts.[13,14] In brief, hematoxylin and eosin–stained sections from resection specimens or biopsies from primary tumors were reviewed by a dedicated head and neck pathologist (SMW), who marked areas of tumor tissue. From the formalin-fixed and paraffin-embedded (FFPE) tissue blocks, three representative 0.6 mm cylinders were transferred to a recipient block using the TMA-Grandmaster (3D Histech, Budapest, Hungary). In general, the tissues were taken from resection specimens for OSCC. As LSCC and OPSCC patients were mostly treated with radiotherapy, tissues were mostly derived from biopsies.

Staining was performed by deparaffination and rehydration, followed by blocking of endogenous peroxidase activity using a buffer consisting of 3% H_2O_2 solution in PBS. Antigen retrieval was performed by boiling the slides in a pH 9.0 EDTA buffer for 20 minutes. The Novolink kit (Leica Biosystems, Rijswijk, the Netherlands) was used according to the manufacturer's instructions for staining. Incubation with the primary antibody (Mouse-anti-HIF-1a, BD biosciences, cat# 610959, lot 4 073 775, diluted 1:50 in PBS-BSA) was performed overnight at four degrees. During every staining procedure, the same positive control tissue sample (renal cell carcinoma) was stained and the staining pattern was compared to ensure similarity of the staining. The same tissue was used as a negative control, by incubation with PBS-BSA instead of the primary antibody.

Scoring was performed visually by an experienced head and neck pathologist (SMW) and ENT-resident (JES) in consensus, blinded to the patient IDs and clinical outcome data. Only nuclear staining of HIF-1a was regarded as biologically relevant and was scored as a percentage expression of tumor cells. A score of 15% or more was considered positive in accordance with our previous study.[15] Cores were regarded as missing if more than half of the core was lost during processing or if a core contained less than 25% tumor tissue.

Sub-analysis of HIF-1a expression in biopsies vs resection specimens

A subgroup of 180 OSCC patients had tissue from both the tumor center and the tumor periphery available for analysis (paired samples). These tissues were used to assess

whether an observed difference between OSCC and LSCC or OPSCC is caused by a different tissue origin (biopsy vs resection specimen) or due to an actual difference between sites. HIF-1a staining was performed in tumor center and tumor periphery tissue. These samples were tested for concordance using a McNemar test.

Data analysis

All analyses were performed using SPSS (IBM, version 25.0). For analysis of immunohistochemical staining, a mean was calculated of the available cores. Baseline data were compared using chi-square analysis for categorical data, Student's *t*-tests for normally distributed data (assessed using Kolmogorov-Smirnov test), and the independent-samples median test for non-normally distributed data. Analysis of variables related to HIF-1a positivity was investigated using logistic regression.

Missing data handling

Missing values for all investigated variables were handled using multiple imputation with 15 datasets. This method of handling missing data has shown increased accuracy compared to performing only a complete case analysis.[16,17] Please find **Supplementary Table ST 1** for an overview of missing data and further details on imputation.

Survival analyses

For survival analyses, Kaplan-Meier analysis (including Log-rank tests), univariate and multivariate Cox-regression were used. Regression analyses were performed in each of the 15 multiple imputed datasets. The presented results were pooled automatically in SPSS, unless stated otherwise. Variables that were significant on univariate Cox-regression were included in the multivariate analysis. HIF-1a expression was included in all multivariate analyses based on clinical relevance. Multivariate analysis was performed per site. We analyzed effect modification by introducing interaction variables into the analyses when appropriate. If the interaction variable had a significant relation to the outcome, this factor was considered an effect modifier and split analyses were performed for each category of the effect modifier.

The balance between the degrees of freedom and the survivors and deceased was no lower than 10 patients per degree of freedom (in the OSCC cohort 391 patients with 45% deceased and thus a maximum of 17° of freedom, in OPSCC: 302 patients with 45% survivors and thus a maximum of 13° of freedom, in LSCC: 248 patients with 33% deceased and thus a maximum of 8° of freedom). A stepwise backward method was used to establish the Cox-regression model of the survival outcomes, by excluding variables with a *P*-value of more than 0.157 (Akaikes Information Criterion).[18]

The following outcomes were analyzed:

Overall survival (OS): An event was defined as death by any cause (time between date of first positive biopsy and date of death). Patients were censored if they were alive at the end of follow-up (time between date of first positive biopsy and date that the patient was last confirmed alive).

Locoregional control (LRC): An event was defined as the occurrence of local and/or regional disease after therapy (time between date of first positive biopsy and date of histological, radiological and/or clinically evident disease). Patients were censored if they did not have a locoregional recurrence at the last follow-up visit, or at the time of death.

Disease-free survival (DFS):. An event was defined as the occurrence of local, regional, or distant disease after therapy (time between date of first positive biopsy and time of histological or clinically evident disease) or death (time between date of first positive biopsy and date of death). Patients were censored if they were alive and free of disease at the end of follow-up (time between date of first positive biopsy and last follow-up that included full ENT-examination)

Disease-specific survival data were not available for all patients and could not be analyzed. Instead we performed a separate OS analysis where the duration of follow-up was cut off at 60 months of follow-up. This cut-off point was chosen as we would expect that most disease-related death would occur in the first 5 years after disease onset. Later deaths may also be explained by second primary tumors or non-disease-related causes of death.

Results

Clinical characteristics

A total of 941 patients were included in the study (**Table 1**): 248 patients with a LSCC, 391 patients with an OSCC, and 302 patients with an OPSCC. Most patients were male and had a history of smoking and alcohol use. For LSCC and OSCC, most tumors were early stage (T1 or T2). In contrast, more than half of patients with an OPSCC were seen with an advanced stage primary tumor or with neck metastases. For the OPSCC cohort, HPV-status was positive in 15.9%. Most tumors were of moderate differentiation grade. After a median of 66 months of follow-up, approximately half of patients were alive.

Variable	Larynx n = 248	Oral Cavity n = 391	Oropharynx n = 302	Total n = 941
Sex				
Male	202 (81.5)	240 (61.4)	212 (70.2)	654 (69.5)
Female	46 (18.5)	151 (38.6)	90 (29.8)	287 (30.5)
Age	66.0 (9.7)	62.1 (11.8)	59.3 (9.0)	62.3 (10.7)
Smoking				
Active or Quit	232 (94.3)	263 (67.6)	270 (90.0)	765 (81.8)
Never	14 (5.7)	126 (32.4)	30 (10.0)	170 (18.2)
Alcohol use				
Active or Quit	188 (78.7)	204 (52.6)	257 (86.2)	649 (70.2)
Never	51 (21.3)	184 (47.4)	41 (13.8)	276 (29.8)
Clinical T-classification				
$cT1_{a/b}$	108 (43.5)	129 (33.0)	36 (12.0)	273 (29.0)
cT2	63 (25.4)	173 (44.2)	89 (29.6)	325 (34.6)
cT3	49 (19.8)	19 (4.9)	68 (22.6)	136 (14.5)
$cT4_{a/b}$	28 (11.3)	70 (17.9)	108 (35.9)	206 (21.9)
Clinical N-classification				
cN0	212 (85.5)	304 (77.7)	106 (35.3)	622 (66.2)
cN1	12 (4.8)	46 (11.8)	48 (16.0)	106 (11.3)
$cN2_{a/b/c}$	24 (9.7)	41 (10.5)	132 (44.0)	197 (21.0)
cN3	0 (0)	0 (0)	14 (4.7)	14 (1.5)
TNM-stage				
Stage I	106 (42.7)	119 (30.4)	16 (5.4)	241 (25.7)
Stage II	53 (21.4)	140 (35.8)	36 (12.0)	229 (24.4)
Stage III	55 (17.7)	42 (10.7)	57 (19.1)	143 (15.2)
Stage IVA	45 (18.1)	90 (23.0)	158 (52.8)	293 (31.2)
Stage IVB	0 (0)	0 (0)	32 (10.7)	32 (3.4)
HPV-status				
Positive	NA	NA	46 (15.9)	NA
Differentiation grade				
Well	10 (9.4)	33 (8.6)	4 (1.7)	47 (6.5)
Moderate	76 (71.7)	298 (77.6)	162 (70.1)	536 (74.3)
Poor	20 (18.9)	53 (13.8)	65 (28.1)	138 (19.1)
Primary treatment				
Surgery	61 (24.6)	199 (51.0)	16 (5.3)	276 (29.4)
Surgery + PORT	40 (16.1)	191 (49.0)	96 (31.8)	327 (34.8)
Surgery + POCRT	3 (1.2)	0 (0)	5 (1.7)	8 (0.9)
Radiotherapy	137 (55.2)	0 (0)	74 (24.5)	211 (22.4)
Chemoradiation	7 (2.8)	0 (0)	111 (36.8)	118 (12.6)
HIF-1a expression				
Median % (IQR)	25.7 (46.6)	39.9 (42.5)	23.9 (45.0)	30.1 (45.0)
Positive (> 15%)	147 (64.2)	290 (76.9)	164 (57.1)	601 (67.3)
Negative (≤ 15%)	82 (35.8)	87 (23.1)	123 (42.9)	292 (32.7)
Survival				
Alive (%)	163 (65.7)	215 (55.0)	135 (44.7)	513 (54.5)
Median months of follow-up for surviving patients	60.0	79.0	66.0	66.0

Table 1 *(previous page)* | Baseline patient characteristics according to site NA: not applicable, PORT postoperative radiotherapy, POCRT postoperative chemoradiotherapy, IQR interquartile range.

Categorical variables are shown as n (%), normally distributed data are shown as mean (SD), non-normally distributed, continuous data are shown as median (IQR). Some baseline characteristics were not available and were scored as missing, this is further shown in **Supplementary Table ST 1**.

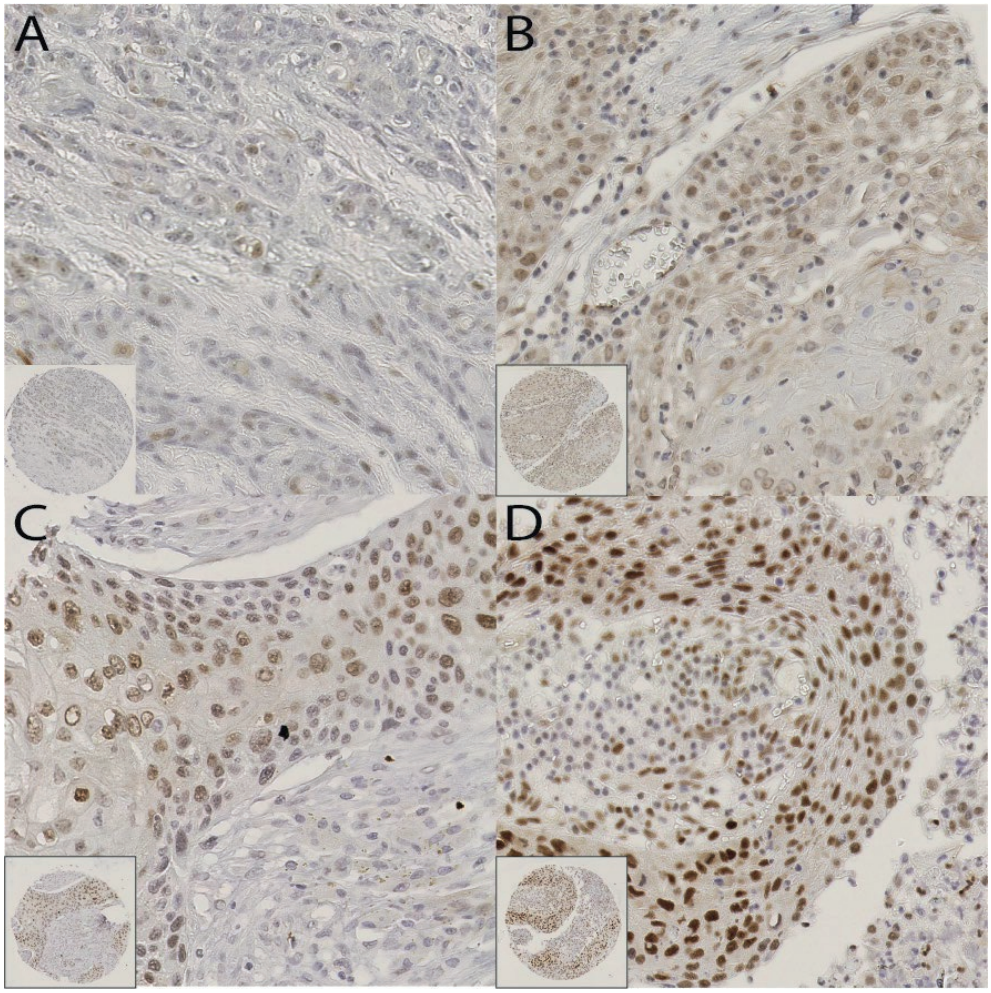

Figure 1 | Example of HIF-1a staining. Examples of immunohistochemical staining for HIF-1a. A. LSCC TMA core. One percent of tumor cells showed positive nuclear staining for HIF-1a. B. OSCC TMA core with 10% HIF-1a positivity. C. OPSCC TMA core with 50% HIF-1a positivity. D. OPSCC TMA core with 90% HIF-1a positivity.

HIF-1a expression is correlated to site

An example of the HIF-1a staining can be found in **Figure 1**. HIF-1a expression was significantly different between sites (p< .001, independent samples median test). Expression of HIF-1a was significantly higher in patients with an oral cancer (median 39.9% positive cells), compared to oropharyngeal (median 23.9%) and laryngeal cancer (median 25.7%). Positivity for HIF-1a (more than 15% HIF-1a positive cells) was present in 290 (76.9%) OSCC patients, 146 (64.2%) LSCC patients, and 164 (57.1%) OPSCC patients. There was no correlation between HIF-1a expression and either T-classification, N-classification, differentiation grade or HPV-status.

HIF-1a expression is associated with poor OS in patients with laryngeal and oropharyngeal, but not in oral squamous cell carcinomas

Age, smoking, clinical T-classification and N-classification, site, HPV-status, differentiation grade, and choice of treatment were significantly related to OS (**Table 2**). When analyzed in the whole cohort, HIF-1a expression was not related to OS (**Figure 2**). However, there was significant effect modification between tumor site and HIF-1a expression: there was a significant difference in the effect of HIF-1a expression on OS between the different sites. HIF-1a expression was associated with worse OS in LSCC and OPSCC. In contrast, HIF-1a expression was associated with better OS in patients with OSCC, although this difference did not reach statistical significance. In a subgroup analysis of HPV-associated OPSCC, HIF-1a positivity was a significant predictor of worse survival with a hazard ratio (HR) of 5.47 (95% CI: 1.16-25.8).

Treatment strategy was a significant univariate predictor of OS in OSCC with a HR of 0.57 (95% CI 0.42-0.77, p< .001) for surgery only vs surgery with postoperative radiotherapy (PORT). It was also a significant predictor in OPSCC with a HR of 0.50 (95% CI 0.23-1.08, p=.079) for surgery vs CRT, a HR of 0.54 (95% CI 0.38-0.78, p=.001) for surgery with PORT vs CRT, HR of 0.89 (95% CI 0.28-2.82, p=.84) for surgery with postoperative CRT vs CRT, and HR of 0.58 (95% CI 0.39-0.89, p=.011) for primary RT vs CRT. There was no significant difference between treatment strategies in LSCC.

Because of the site difference, multivariate analyses were performed separately for each tumor site (**Table 3**). In LSCC, HIF-1a expression retained a significant association with

Variable	Cohort	Overall survival	
		HR (95% CI)	*p*
Age	Overall		
Per year increase		1.02 (1.02 – 1.03)	<0.001
Sex	Overall		
Female vs. male		0.94 (0.76 – 1.15)	NS
Alcohol	Overall		
Active/quit vs never		1.17 (0.94 – 1.45)	NS
Smoking	Overall		
Active/quit vs never		1.41 (1.07 – 1.85)	0.014
Clinical T-classification	Overall		
cT3-4 vs cT1-2		2.53 (2.08 – 3.04)	<0.001
Clinical N-classification	Overall		
cN+ vs cN0		2.57 (2.13 – 3.11)	<0.001
Site	Overall		
Larynx vs oropharyngeal		0.58 (0.45 – 0.76)	<0.001
Oral vs oropharyngeal		0.68 (0.55 – 0.84)	<0.001
HPV-status*	Overall		
Positive vs negative		0.27 (0.14 – 0.52)	<0.001
Differentiation grade	Overall		
moderate vs well		1.84 (1.07 – 3.17)	0.028
poor vs well		1.80 (0.97 – 3.31)	NS
HIF-1a			
positive vs negative	Overall	1.14 (0.92 – 1.41)	NS
	Within larynx†	1.86 (1.10 – 3.14)	0.020
	Within oral cavity†	0.77 (0.55 – 1.09)	NS
	Within oropharynx†	1.46 (1.05 – 2.02)	0.023
Treatment	Overall		
Surgery + PORT vs surgery		1.82 (1.42 – 2.35)	<0.001
Surgery + POCRT vs surgery		3.01 (1.90 – 4.77)	0.017
Primary RT vs surgery		1.45 (1.07 – 1.97)	0.016
Primary CRT vs surgery		2.94 (2.16 – 4.00)	<0.001

Table 2 | Univariate survival analyses. Abbreviations: HR hazard ratio, CI confidence interval, NS not significant (p>0.05), RT radiotherapy, PORT postoperative radiotherapy, POCRT postoperative chemoradiotherapy, CRT chemoradiotherapy. †: Within analyses were performed by including the interaction variable HIF-1a*Site, not by a subgroup analysis

* Oropharyngeal cancer only

† Within analyses were performed by including the interaction variable HIF-1a*Site, not by a subgroup analysis

Variable	Locoregional control		Disease-free survival	
	HR (95% CI)	*p*	HR (95% CI)	*p*
Age				
Per year increase	1.00 (0.99 – 1.02)	NS	1.03 (1.02 - 1.04)	<0.001
Sex				
Female vs. male	1.08 (0.78 – 1.49)	NS	1.00 (0.82 – 1.22)	NS
Alcohol				
Active/quit vs never	0.97 (0.70 – 1.35)	NS	1.20 (0.98 - 1.48)	NS
Smoking				
Active/quit vs never	0.77 (0.54 – 1.11)	NS	1.32 (1.03 - 1.70)	0.031
Clinical T-classification				
cT3-4 vs cT1-2	1.65 (1.22 – 2.24)	0.001	2.10 (1.75 - 2.52)	<0.001
Clinical N-classification				
cN+ vs cN0	1.83 (1.35 – 2.49)	<0.001	2.10 (1.75 - 2.52)	<0.001
Site				
Larynx vs oropharyngeal	0.76 (0.51 – 1.14)	NS	0.82 (0.64 - 1.04)	NS
Oral vs oropharyngeal	0.73 (0.52 – 1.03)	NS	0.68 (0.55 - 0.83)	<0.001
HPV-status				
Positive vs negative	0.40 (0.16 – 1.00)	NS	0.39 (0.22 - 0.68)	0.001
Differentiation grade				
moderate vs well	2.16 (0.81 – 5.74)	NS	1.68 (0.97 - 2.91)	NS
poor vs well	2.54 (0.95 – 6.82)	NS	1.74 (0.98 – 3.10)	NS
HIF-1a				
positive vs negative overall	1.13 (0.81 – 1.59)	NS	1.04 (0.85 - 1.27)	NS
Within larynx†	1.76 (0.81 – 3.79)	NS	1.72 (1.08 – 2.73)	0.022
Within oral cavity†	0.77 (0.44 – 1.34)	NS	0.72 (0.51 – 1.00)	0.049
Within oropharynx†	1.41 (0.84 – 2.37)	NS	1.30 (0.96 – 1.77)	NS
Treatment				
Surgery + PORT vs surgery	1.50 (1.00 – 2.26)	0.049	1.42 (1.11 – 1.81)	0.005
Surgery + POCRT vs surgery	2.07 (0.50-8.59)	NS	2.54 (1.03 – 6.24)	0.043
Primary RT vs surgery	1.69 (1.07 – 2.64)	0.023	1.59 (1.20 – 2.10)	0.001
Primary CRT vs surgery	2.26 (1.38 – 3.70)	0.001	2.49 (1.85 – 3.33)	<0.001

Table 2 (*continued*) |

reduced OS independent of age, T-classification and N-classification. In OPSCC, HIF-1a expression retained a significant association with reduced OS independent of age, T-classification and N-classification, HPV-status, and treatment modality. In OSCC, age, T-classification and N-classification, and differentiation grade were significantly associated with OS. Also in the multivariate analysis in OSCC, HIF-1a was associated with better survival, but the difference did not reach statistical significance.

When OS time was cut off at 60 months the results were mostly in concurrence with the overall survival analysis (**Supplementary Table ST 2**). However in this analysis, we found a statistically significant better survival in HIF-1a positive OSCC.

To further investigate the site differences, we investigated several variables for confounding or effect modification in the relation between site and survival. These variables were treatment strategy, smoking history, and alcohol history. None of these variables were significant effect modifiers or confounders in the relation between HIF-1a expression and survival. Site was the only effect modifier. Moreover, treatment strategy was not a significant

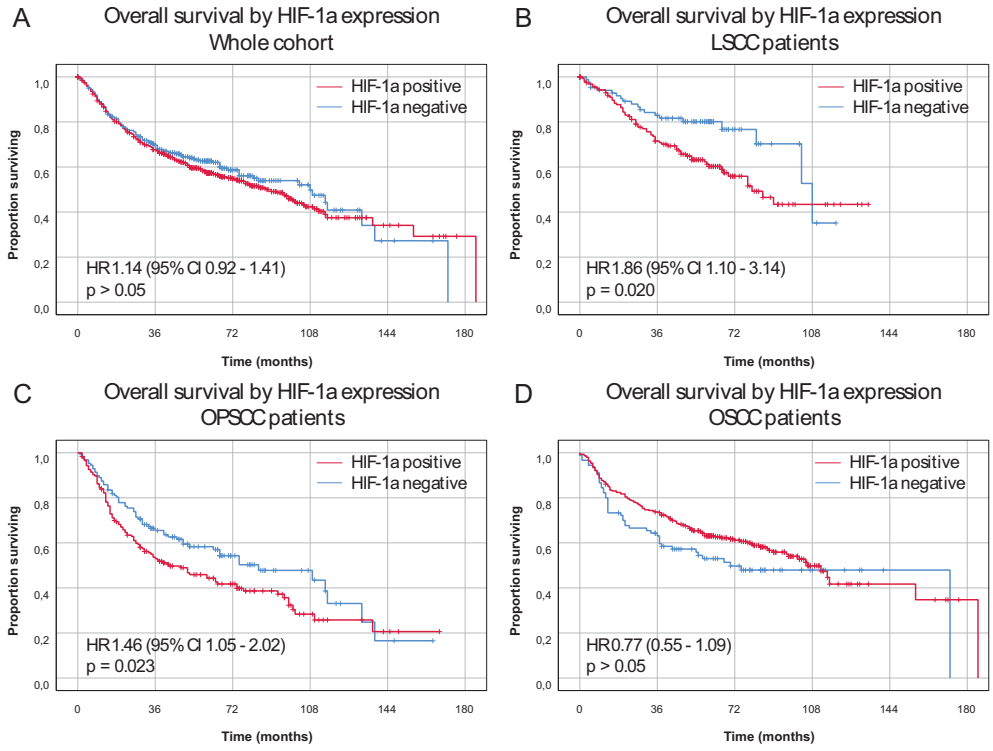

Figure 2 | Overall survival by HIF-1a expression. In LSCC and OPSCC patients, HIF-1a positive tumors show significantly worse survival. In the OSCC patient cohort, HIF-1a positive tumors appear to have better survival, but this is not statistically significant.

effect modifier in the relation between HIF-1a expression and survival overall and within each site. This suggests that in all treatment strategies, HIF-1a expression was associated with better (OSCC) or worse (LSCC, OPSCC) survival.

No association of HIF-1a expression and LRC

T-classification and N-classification as well as treatment modality predicted LRC. HIF-1a expression was not related to LRC. Therefore, no further analyses were performed.

HIF-1a expression is associated with poor DFS in LSCC and OPSCC and improved DFS in OSCC

DFS analyses were mostly concurrent with OS analyses. The location of recurrence per site is shown in Table S3. In univariate analyses, age, smoking, clinical T-classification and N-classification, site, HPV-status, and treatment were significant predictors of DFS (Table 2). As with OS, there was effect modification caused by the site of the primary tumor: the effect of HIF-1a expression on DFS was significantly different among sites. In LSCC and OPSCC, HIF-1a positivity was associated with worse DFS, although this difference did not reach statistical significance in OPSCC. In OSCC, HIF-1a positivity was associated with significantly better survival DFS.

The significant effect of HIF-1a positivity in LSCC and OSCC remained statistically significant in multivariate analyses with age, clinical T-classification and N-classification, grade (OSCC only), and treatment strategy (LSCC only), as seen in Table 4. In multivariate analysis in OPSCC, there was no statistically significant effect of HIF-1a positivity for DFS.

Concordance between tumor center and periphery in OSCC

There was no significant discordance between samples taken from the tumor center or tumor periphery in the 180 OSCC patients that had paired samples available (McNemar test $P = 1.00$). Moreover, in these patients HIF-1a positivity in the tumor center and tumor periphery were both associated with better OS, although the difference did not reach statistical significance on univariate survival analysis in this smaller subgroup

Variable	Laryngeal cancer		Oral cancer		Oropharyngeal cancer	
	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*
Age						
Per year increase	1.06 (1.03 – 1.08)	<0.001	1.04 (1.03 – 1.06)	<0.001	1.03 (1.01 – 1.05)	0.005
Smoking						
Active/quit vs never	NA	NA	NA	NA	NA	NA
Clinical T-classification						
cT3-4 vs cT1-2	2.06 (1.29 – 3.29)	0.003	1.71 (1.23 – 2.38)	0.002	1.80 (1.21 – 2.69)	0.004
Clinical N-classification						
cN+ vs cN0	2.96 1.73 – 5.04)	<0.001	3.03 (2.14 – 4.29)	<0.001	2.18 (1.51 – 3.16)	<0.001
HPV-status						
Positive vs negative	NA	NA	NA	NA	0.24 (0.13 – 0.47)	<0.001
Differentiation grade						
moderate vs well	NA	NA	2.01 (0.97 – 4.13)	NS	NA	NA
poor vs well			2.25 (1.01 – 5.01)	0.047		
HIF-1a						
positive vs negative	1.72 (1.02 – 2.89)	0.041	0.71 (0.50 – 1.01)	NS	1.46 (1.04 – 2.05)	0.029
Treatment *Larynx*	NA	NA				
Treatment *Oral Cavity*			NA	NA		
Treatment* *Oropharynx*						
Surgery vs CRT					0.92 (0.40-2.10)	NS
Surgery + PORT vs CRT					0.58 (0.36 – 0.93)	0.024
Surgery + POCRT vs CRT					0.82 (0.24 – 2.79)	NS
Radiotherapy alone vs CRT					1.10 (0.67 – 1.79)	NS

Table 3 | Final multivariate models for overall survival Abbreviations: HR hazard ratio, CI confidence interval, NS not significant (p>0.05), CRT chemoradiotherapy, PORT postoperative radiotherapy, POCRT postoperative chemoradiotherapy. NA: HPV was only included in oropharyngeal cancer analyses, other variables are reported NA if they were removed from the model by backward selection using Akaike's information criterion (p>0.157) for removal. *: Treatment: the largest category was chosen as reference category for each site.

Variable	Laryngeal cancer		Oral cancer		Oropharyngeal cancer	
	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*
Age						
Per year increase	1,03 (1,01 - 1,06)	0,004	1,04 (1,03 - 1,06)	<0.001	1,02 (1 - 1,04)	0,018
Smoking						
Active/quit vs never	NA	NA	NA	NA	NA	NA
Clinical T-classification						
cT3-4 vs cT1-2	2,18 (1,32 - 3,62)	0,002	1,46 (1,05 - 2,02)	0,023	1,71 (1,18 - 2,48)	0,005
Clinical N-classification						
cN+ vs cN0	3,22 (1,87 - 5,55)	<0.001	2,63 (1,88 - 3,68)	<0.001	1,83 (1,29 - 2,6)	0,001
HPV-status						
Positive vs negative	NA	NA	NA	NA	0,35 (0,20 - 0,63)	<0.001
Differentiation grade						
moderate vs well	NA	NA	2.29 (1.11 – 4.71)	0.024	NA	NA
poor vs well			2.17 (0.97 – 4.83)	NS		
HIF-1a						
positive vs negative	1,89 (1,16 - 3,07)	0,011	0,65 (0,46 - 0,91)	0,012	1,32 (0,96 - 1,82)	0,085
Treatment* *Larynx*						
Surgery vs RT	0,64 (0,37 - 1,13)	NS				
Surgery + PORT vs RT	0.32 (0.16 – 0.62)	0.001				
Surgery + POCRT vs RT	0.43 (0.10 – 1.97)	NS				
CRT vs RT	0.57 (0.16 – 2.03)	NS				
Treatment *Oral Cavity*			NA	NA		
Treatment* *Oropharynx*						
Surgery vs CRT					0,84 (0,37 - 0,91)	NS
Surgery + PORT vs CRT					0.55 (0.37 – 0.81)	0.002
Surgery + POCRT vs CRT					0.80 (0.25 – 2.55)	NS
RT alone vs CRT					0.93 (0.59 – 1.48)	NS

Table 4 | Final multivariate models for disease-free survival. Abbreviations: HR hazard ratio, CI confidence interval, NS not significant (p>0.05), RT radiotherapy, PORT postoperative radiotherapy, POCRT postoperative chemoradiotherapy, CRT chemoradiotherapy. NA: HPV was only included in oropharyngeal cancer analyses, other variables are reported NA if they were removed from the model by backward selection using Akaike's information criterion (p>0.157) for removal. * Treatment: the largest category was chosen as reference category for each site.

Discussion

This study compared the effect of HIF-1a on OS, LRC, and DFS in 941 patients with a squamous cell carcinoma in the three major sites in HNSCC: the oral cavity, oropharynx, and larynx. High HIF-1a expression was observed more often in OSCC as compared to the other two sites. In LSCC and OPSCC, HIF-1a expression was associated with worse OS and DFS, but with better OS and DFS in OSCC. The association between HIF-1a expression and survival was independent of other known prognosticators such as T-classification, N-classification, and HPV-status. These findings underline biological differences between squamous cell tumors in the head and neck region.

HIF-1a is a protein that is continuously synthesized, but also degraded under normoxic conditions. As this degradation process is oxygen-dependent, hypoxia leads to accumulation of HIF-1a, activating transcription of its downstream targets. This activates cellular survival mechanisms that enable cells to survive under hypoxic conditions. In the case of cancer cells, these survival mechanisms also improve resistance to radiotherapy and some forms of chemotherapy.[19] HIF-stabilization can also occur by oncogenic activation, independent of hypoxia.[20,21] In most studies within HNSCC, high HIF-1a expression is associated with worse survival in each of the major sites, including OSCC.[3]

Interestingly, there are several studies that describe an association of high HIF-1a expression with better survival and these findings were all in OSCC.[22–24] It is suggested in these studies that the high expression of HIF-1a is caused by oncogenic activation. The transcription of the downstream targets, such as VEGF, then improves vascularity and oxygen availability. This in turn is suggested to increase the efficacy of (post-operative) radiotherapy, improving survival. While this is a viable explanation of how high HIF-1a expression is associated with improved survival, this fails to explain why this phenomenon only occurs in OSCC and not in the other sites, which are more often treated with radiotherapy. However, it does concur with our finding that high HIF-1a expression leads to worse survival in OPSCC and LSCC, but not in OSCC. We also investigated the effect of HIF-1a expression on survival in patients who underwent surgery vs organ preservation therapy independent of site and found no differences between treatment strategies. We therefore conclude that the differences in the relation between HIF-1a expression and survival are to be attributed to the site and not the treatment strategy. It can be hypothesized that in OSCC the oncogenic activation of HIF-1a plays a major role in HIF-1a expression, while in the other sites, hypoxia is the major driver of HIF-1a stabilization, illustrating the heterogeneity between tumors of different sites.

In addition, we found that HIF-1a expression only had a significant effect of the survival outcomes OS and DFS, but not LRC. This suggests that the effect of HIF-1a expression on

survival may occur through distant metastasis. It has been described that hypoxia may cause hypoxia-induced proteome changes, causing delayed recurrences or dormant micrometastasis.[25]

Site differences are immediately observed when performing clinical staging according to the TNM system.[26] In the oral cavity, tumor size and depth of invasion are the criteria for staging of oral or oropharyngeal tumors, while in the larynx tumors are considered of a higher stage when invading multiple anatomical subregions, independent of gross tumor size. Treatment strategy, that is, the choice of a primary surgical or (chemo-)radiation approach, also differs per site. However, this difference has mostly to do with functionality and the best chance of eradicating disease while maintaining function.

In the present study, the proportion of patients who had ever smoked or had used alcohol was lower in the OSCC patients than in the LSCC and OPSCC patients. We did not find significant effect modification of smoking or drinking status on the relation between HIF-1a and survival. Still, it is possible that this has led to other, unknown differences in tumor biology or etiology that have contributed to the observed differences.

HPV-associated (HPV+) OPSCC is a subgroup that has much better survival than non-HPV associated (HPV−) OPSCC. We found that HIF-1a expression is associated with worse survival even in the subgroup of HPV+ tumors. Still the overall OS and DFS in the whole group of OPSCC patients were relatively low. This can be explained by the relatively low percentage of HPV+ OPSCC in our country, as compared to countries such as the United States.[27,28] The observed adverse survival effect of HIF1a expression, independent of HPV status is worthy of confirmation in other OPSCC cohorts.

Treatment strategy was a significant predictor of survival in LSCC (DFS), OSCC (OS), and OPSCC (OS, DFS) in univariate analysis. In OSCC, patients treated with surgery and PORT had worse survival than patients treated with surgery only. In multivariate analysis, controlling for other variables, treatment strategy was no longer related to survival in OSCC. Therefore, we conclude that the univariate survival difference between surgery and surgery + PORT is related to the tumor characteristics that lead to the indication for PORT.

Several points concerning the present study should be taken into account. HIF-1a staining and scoring was performed using the same staining protocol and in a TMA; therefore, tumors from different sites and different tumor sizes could be scored in a similar way and were scored by the same pathologist. It has been shown that TMA analysis with three or more spots from the same tumor yields a representative sample of the tumor and this has been shown to be reliable for high-throughput molecular profiling.[13,29–32] In addition, specifically for HIF-1a staining, a previous study shows that single-core TMAs have a good concurrence with full sections and that false-positivity is rare.[33] We believe that by using triplicate cores concurrence will be comparable or better. The use of TMAs makes the

present study one of the largest single-center studies on the effect of HIF-1a expression on survival. All patients were treated by the same team of physicians and treatment decisions made in the same MDT.

In addition, it could be argued that the site difference between OSCC and LSCC and OPSCC could be explained because of the tissue origin; OPSCC and LSCC tissues were mostly derived from biopsies, while in most OSCC patients the analyzed tissues originated from resection specimens. To investigate this, we performed sensitivity analyses in a subgroup of OSCC patients that had tissue taken from the tumor center and the tumor periphery (which could be considered a location where a biopsy might be taken). Because there was no significant discordance in HIF-1a positivity between these samples and because the observed effect was similar in these samples (improved survival in HIF-1a positive OSCC patients), we conclude that the observed outcome is not due to a difference in tissue origin, that is, biopsy or resection specimen.

This study was retrospective by design and patients were included in different cohorts. Some patients had undergone biopsy in other hospitals, before being referred to our tertiary hospital. The tissues of these patients were not always available for analysis. However, apart from this we included all consecutive patients who were treated in our hospital. Because no patients had been included or excluded based on either the outcome (survival) or the determinant (HIF-1a) expression, we do not believe that substantial bias was introduced. Moreover, the year of treatment differed between cohorts. Unfortunately because the data had been anonymized, it could not be investigated whether the year of treatment has had a direct effect on survival. However, we do not find it likely that the year of treatment confounds the relation between site, HIF-1a expression and survival.

In conclusion, we found that HIF-1a expression is significantly different across sites within HNSCC. Moreover, HIF-1a overexpression had a significantly different effect on patient outcomes between sites. Tumor hypoxia is a target for therapy: hypoxia radiosensitizers, such as nimorazole in conjunction with primary radiotherapy or ARCON therapy, are available to increase susceptibility to radiotherapy.[34,35] Also, drugs that directly inhibit HIF-1a are currently being investigated as anticancer treatment.[36] If HIF-1a is considered to be a marker of hypoxia in OPSCC and LSCC, particularly patients with tumors of these sites will benefit from a hypoxia radiosensitizer. In OSCC, high expression of HIF-1a may be caused by oncogenic activation, which improves vascularity and oxygen availability. These tumors will less likely benefit from these therapy modalities. The present study highlights biological differences between HNSCC sites and warrants further investigation into these differences, rather than treating HNSCC as a single entity.

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Supplementary information S1

Post-operative (chemo)radiotherapy was advised if at least one of the following criteria were present, and the patient was below 70 years of age and sufficiently fit for chemoradiotherapy (in which case only postoperative radiotherapy was advised). Concomitant chemotherapy to adjuvant radiotherapy was added since 2005.

- Extranodal extension of lymph node metastasis in neck dissection specimen
- Irradical resection / positive margins: margin < 1 mm
- Post-operative radiotherapy was advised if at least one of the following criteria were present on final pathological examination:
- N2b neck or higher

Post-operative radiotherapy was advised if three or more of the following criteria were present on final pathological examination:

- Perineural growth
- Angioinvasive growth
- Spidery tumor growth (rather than pushing tumor borders)
- Close resection margins: margin 1-5 mm

Supplementary Table ST 1. Missing data handling

The percentage of cases with at least one missing value was 39.4%. If HPV is considered not-missing in patients with non-oropharyngeal cancer, this percentage is 29.5%.

Variable	N missing (%)	Impute?	Predictor	Constraints
Sex	0 (0%)	No	Yes	
Age	12 (1.3%)	Yes	Yes	23-91*
Smoking	6 (0.6%)	Yes	Yes	
Alcohol use	16 (1.7%)	Yes	Yes	
Clinical T-stage	1 (0.1%)	Yes	Yes	
Clinical N-stage	2 (0.2%)	Yes	Yes	
Treatment	0 (0%)	No	Yes	
Differentiation grade	220 (23.4%)	Yes	Yes	
Presence of HPV†		Yes	Yes	
Total number of patients	244 (25.9%)			
Oropharyngeal cancer	12 (3.9%)			
Synchronous primary tumor	2 (0.2%)	Yes	Yes	
Subsite	0 (0%)	No	Yes	
Recurrence yes/no	4 (0.4%)	No	Yes	
Recurrence time	12 (1.3%)	No	Yes	
Survival time (months)	2 (0.2%)	Yes	Yes	0-185*
Death yes/no	0 (0%)	No	Yes	
HIF-1a positive/negative	48 (5.1%)	Yes	Yes	

* For continuous variables, the observed minimum and maximum values were used as constraints. † HPV-status had been determined in most patients. After imputing, HPV was recoded into "positive", "negative" and "non-oropharyngeal cancer"

Imputation Script

```
MULTIPLE IMPUTATION Sex Age Smoking_dich Alcohol_dich cT_Dich cN_dich
TX_BSL Path_Differentiation HPV_HR Synch_Prim Subsite LRC_Outcome
LRC_Months OS_Months OS_Outcome HIF_15
  /IMPUTE METHOD=AUTO NIMPUTATIONS=15 MAXPCTMISSING=NONE
   MAXCASEDRAWS=100 MAXPARAMDRAWS=100
  /CONSTRAINTS Sex( ROLE=IND)
  /CONSTRAINTS Age( MIN=23.0 MAX=91.0 RND=1.0)
  /CONSTRAINTS TX_BSL( ROLE=IND)
  /CONSTRAINTS Subsite( ROLE=IND)
  /CONSTRAINTS LRC_Outcome( ROLE=IND)
  /CONSTRAINTS LRC_Months( ROLE=IND)
  /CONSTRAINTS OS_Months( MIN=0.0 MAX=185.0)
  /MISSINGSUMMARIES NONE
  /IMPUTATIONSUMMARIES MODELS
  /OUTFILE IMPUTATIONS='filename'.
```

Supplementary Table ST 2. Final multivariate models overall survival (cutoff at 60 months)

	Laryngeal cancer		Oral cancer		Oropharyngeal cancer	
	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*
Age						
Per year increase	1.05 (1.02 – 1.08)	< 0.001	1.04 (1.03 – 1.06)	< 0.001	1.03 (1.01-1.05)	0.005
Smoking						
Active/quit vs never	NA	-	NA	-	NA	-
Clinical T-classification						
cT3-4 vs cT1-2	2.26 (1.30 – 3.96)	0.004	1.63 (1.14 – 2.33)	0.007	1.81 (1.18 – 2.80)	0.007
Clinical N-classification						
cN+ vs cN0	3.08 (1.72 – 5.52)	< 0.001	3.61 (2.49 – 5.22)	< 0.001	1.96 (1.31 – 2.93)	0.001
HPV-status						
Positive vs negative	NA	-	NA	-	0.31 (0.16 – 0.60)	0.001
Differentiation grade						
moderate vs well			2.69 (1.09 – 6.65)	0.032		
poor vs well	NA	-	3.10 (1.17 – 8.26)	0.023	NA	-
HIF-1a						
positive vs negative	2.07 (1.13 – 3.78)	0.018	0.59 (0.40 – 0.85)	0.005	1.52 (1.05 – 2.18)	0.025
Treatment *Larynx*						
Surgery vs Primary RT	0.44 (0.21 – 0.94)	0.034				
Surgery + PORT vs Primary RT	0.55 (0.28 – 1.07)	NS				
Surgery + POCRT vs Primary RT	0.78 (0.17 – 3.58)	NS				
Primary CRT vs Primary RT	0.37 (0.08 – 1.68)	NS				
Treatment *Oral Cavity*						
Surgery + PORT vs surgery			NA	-		
Treatment						
Surgery vs primary CRT					0.64 (0.24 – 1.73)	NS
Surgery + PORT vs primary CRT					0.56 (0.36 – 0.85)	0.007
Surgery + POCRT vs primary CRT					0.74 (0.23 – 2.39)	NS
Radiotherapy alone vs primary CRT					0.77 (0.45 – 1.31)	NS

A: HPV was only included in OPSCC analyses, other variables NA when removed from the model by backward selection using Akaike's information criterion (p>0.157) criterion for removal. *: Treatment: the largest category was chosen as reference category for each subsite

Chapter 8

Summary discussion and possibilities for future research

Summary discussion

Treatment failure in head and neck squamous cell carcinoma (HNSCC), as is reflected in the relatively low survival rates, remains an important problem.[1,2] It's still elusive why some tumors respond well to therapy, be it surgery, radiotherapy or chemoradiation, and some do not. The answer to these questions may lie in the biology of the tumor, in the constitution of the microenvironment, tumor cell genetics and epigenetics.[3] By gaining more insight to the hallmarks of each individual tumor we hope to identify not only prognostic factors, but also predictive factors for response to therapy, so that the optimal treatment can be selected for each individual patient and tumor.

In the studies described in this dissertation, we have focused on the prognostic effects of tumor hypoxia and how it interacts with other factors in the tumor microenvironment. Tumor hypoxia leads to a decreased sensitivity to radiotherapy and some forms of chemotherapy that require the presence of oxygen to induce maximum damage to tumor cells.[4–7] Moreover, hypoxia triggers pathways inside all nucleated cells in the body (including cancer cells) that activates cellular survival mechanisms.[8,9] These mechanisms increase a cell's potential to withstand treatment, to not go into apoptosis, to increase its potential for metastasis and to evade the immune system.[10]

In **Chapter 1** we have summarized the effects of tumor hypoxia and several methods to detect tumor hypoxia using biomarkers or other techniques. In **Chapter 2** we have performed a cell-based analysis to compare expression of the endogenous hypoxia marker (EMH) HIF-1a to pimonidazole, an exogenous marker of hypoxia that is often considered a gold standard in basic scientific research. Although we found a weak significant correlation between both markers on a tissue fragment level, we did not identify a significant correlation on a whole tissue biopsy level. We concluded that the expression of each hypoxia markers represents a different form of hypoxia and that both markers provide complementary information

In **Chapter 3** we investigated the relation between hypoxia and two other factors of the tumor microenvironment, specifically PD-L1 expression and the level of tumor-infiltrating lymphocyte (TIL) invasion. This was done in a cohort of oropharyngeal squamous cell carcinoma (OPSCC) patients. We found a negative correlation between HIF-1a expression and the level of TIL-invasion, suggesting that hypoxia decreases the number of TILs. Moreover, PD-L1 positivity was correlated to higher number of CD3+ and CD8+ TILs. Hypoxia and low TIL-invasion were both independent prognostic factors for reduced overall survival (OS). These findings highlight interaction of features within the tumor microenvironment. Moreover, it highlights that various features of the microenvironment are independently relevant for clinical patient outcomes.

In **Chapter 4** we investigated the presence of hypoxia and two other factors of the tumor microenvironment: T-cell influx and proliferation in a sample of patients who had also undergone diffusion-weighted magnetic resonance imaging (DW-MRI). This was a pilot study that combined data from the previously described OPSCC cohort and another study that investigated the correlation of ADC and HPV-status.[11] DW-MRI is an MRI-sequence that illustrates the ability for diffusion of water molecules through tissue. A tumor's apparent diffusion coefficient (ADC) may be provided from this MRI sequence. It is thought that this ADC resembles the microarchitecture of a tumor and therefore would reflect histological properties such as cellularity in the apparent diffusion coefficient (ADC). We hypothesized that hypoxia leads to necrosis, leading to increased diffusion of water molecules and therefore a positive correlation to ADC. As T-cell influx and high proliferation rate lead to higher cellularity, we hypothesized a negative correlation with ADC, in line with a previous publication.[12]

The expected correlations were observed, although no statistical significance was reached between HIF-1a expression and ADC in this small cohort. There was a significant correlation between T-cell influx (CD3+ cell count) and ADC as well as proliferation (Ki67 expression) and ADC.

The further studies focused on the effect of hypoxia markers on clinical outcome in HNSCC patients. In **Chapter 5** we performed a literature review on the effect of EMH expression on outcomes of patients with HNSCC. We have identified 40 different manuscripts on the effects of EMH expression in HNSCC patients and found that many different EMHs are used to investigate hypoxia. Interestingly the methods to discern a hypoxic from a non-hypoxic tumor are highly heterogeneous. The cellular compartments scored for each marker (i.e. nuclear, membranous or cytoplasmic staining) and the cutoff value used for positivity, differed highly even between studies on the same EMH. Because of this, performing a meta-analysis was not possible. While the methods varied between studies, the conclusions were often the same: tumors with overexpression of EMHs were often associated with worse outcome than tumors with low expression. Moreover, high EMH expression was also often correlated with adverse clinical characteristics such as a higher T- or N-classification, or poor differentiation. Interestingly, the negative effect on outcome was encountered in both surgical and organ-preservation therapies. So even when excision of the entire tumor and its hypoxic areas is performed, high expression of EMHs was still associated with worse outcomes. This shows that there is a role for hypoxic-modification treatment, or HIF-inhibitors to improve the prognosis of this group of patients

In the following **Chapter 6** we investigated the effect of expression of the EMH HIF-1a and two of its downstream targets, GLUT-1 and CA-IX, on survival in OPSCC. Specifically, this study aimed to compare expression of these hypoxia markers in patients with HPV-positive (HPV+) versus HPV-negative (HPV-) patients, as these are two patient groups with distinct

oncogenic differences. We found that high expression of HIF-1a was associated with worse OS. In a multivariate analysis, this association was independent of T- and N-classification, treatment, HPV-status and age. CA-IX expression was not associated with OS. GLUT-1 expression was associated with OS but was not an independent predictor in the multivariate analysis.

While our literature review had already identified several studies on the effect of HIF-1a expression in OPSCC patients, our study emphasized the relation of HIF-1a expression and HPV-status. We did not find a difference in HIF-1a expression between HPV+ and HPV- OPSCC. However, the effect of HIF-1a expression on survival of patients with HPV+ OPSCC was significantly greater than in HPV- OPSCC. This is interesting as patients with HPV+ tumors are considered a population with an excellent prognosis. However, HIF-1a expression marked a subpopulation of patients with HPV+ tumors with a poor prognosis. In fact, survival of patients with HPV+ OPSCC with high HIF-1a expression was similar to survival of patients with HPV- OPSCC. As de-escalation trials are currently ongoing for patients with HPV+ OPSCC, the identification of this specific subgroup with poor prognosis is especially relevant.[13]

To further investigate differences in biology between different sites of HNSCC, we compared expression of HIF-1a in patients with SCC in the three major sites of HNSCC in a large cohort of patients in **Chapter 7**. We included 391 patients with a SCC of the oral cavity (OSCC), 248 patients with a SCC of the larynx (LSCC) and 301 patients with OPSCC. We found that HIF-1a was much more often expressed in OSCC than in OPSCC and LSCC. High HIF-1a expression was associated with worse OS and DFS in patients with LSCC and OPSCC, but with better OS and DFS in patients with OSCC.

Although all patients with OSCC were treated with surgery with or without adjuvant radiotherapy, an analysis of the effect of HIF-1a expression across all treatment strategies (so regardless of the disease site) did not reveal that the different effect of HIF-1a on prognosis could only be attributed to treatment. In essence, the observed effect of better outcome of HIF-1a expression in OSCC and poor outcome in LSCC and OPSCC was actually caused by the site and not the treatment. While this finding is interesting, it is hard to explain theoretically. It is possible that oncogenic activation, rather than hypoxia, is the main driver for HIF-1a expression in OSCC and that other factors within this activation cascade make the tumors more sensitive to treatment. It can therefore be argued whether patients with OSCC would benefit from treatment with HIF-inhibitors.

Future perspectives

The role of HIF-1a

In summary, the studies in this dissertation have shown that hypoxia and especially HIF-1a expression is relevant in all HNSCC sites, even though there appear to be distinctly different roles for HIF-1a even between sites. This warrants more investigation into the differences in tumor biology between tumors from different anatomical sites. It should be investigated why HIF-1a is more often expressed in OSCC and more importantly why there is improved survival in patients with OSCC that have high HIF-1a expression. Although it is possible that this is due to a different treatment strategy in OSCC versus LSCC and OPSCC (i.e. primary surgical approach vs. primary radiotherapy), this could not be shown from our analysis. It would be interesting to see whether this finding could be validated in a cohort of OSCC patients treated with (chemo-)radiotherapy, although such a study could be hampered by selection bias: usually chemoradiation and radiotherapy are reserved for OSCC patients not fit for surgery or with functional inoperable disease.

Alternatively, it could be that the high expression in this site is merely due to a bystander effect and not through hypoxia. And is there a protective effect, i.e. is the increased HIF-1a expression a result of increase of PI3K pathway activation and does the increased VEGF expression through HIF-1a in this site lead to the formation of effective vascularization and re-oxygenation?[14] Future studies should be performed to investigate the effect of hypoxia in OSCC using both HIF-1a and complementary hypoxia markers such as pimonidazole.

Elucidating the role of HIF-1a driven vascular formation is especially important as VEGF-targeted therapies are currently being investigated.[15] While VEGF targeting will result in decreased number of vessels, there is also evidence that hypervascularization leads to perfusion limited hypoxia.[16,17] Depending on the drug dose, tumors with perfusion limited hypoxia will become normoxic as this hypervascularization is compensated, or will display diffusion limited hypoxia as the number of blood vessels becomes too low for the tumors own blood supply (See also: **Chapter 1**, **Figure 5**). If increased VEGF expression through high HIF-1a levels is beneficial because of vascular normalization, patients with OSCC are less to benefit from VEGF inhibition.

Another role for HIF-1a as a predictive marker has recently been identified in patients with locally advanced HNSCC and were treated with cisplatin-based chemoradiation with or without the epidermal growth factor (EGFR) inhibitor nimotuzumab.[18] Patients with high HIF-1a expression had a benefit of 20 percentage points after 5 years of addition of nimotuzumab for the outcomes locoregional control, progression free survival and overall survival. In current practice, patients with HPV-negative disease who have an indication for treatment with cisplatin but are not eligible to receive this treatment because of an

unacceptable risk of toxicity are often treated with the EGFR inhibitor cetuximab and radiotherapy. The study described above gives rise to the question if there is also a predictive role for HIF-1a expression for these frail patients who are treated with cetuximab. Withholding treatment with cetuximab to potential non-responders spares these patients to 'unnecessary' toxicity.

Interaction of hypoxia and the immune system: the predictive role of HIF-1a

Moreover, the interaction between hypoxia and the immune system that is described in the literature is also present HNSCC. In fact, **Chapter 3** has shown that both these factors are independent prognosticators for patient outcome. As hypoxia has a immunosuppressive effect, it may also influence sensitivity to immunotherapy. A study in renal cell carcinoma has investigated gene expression profiles to discern responders versus non-responders to treatment with axitinib (a tyrosine kinase inhibitor) and pembrolizumab (an anti-PD1 immune checkpoint inhibitor).[19] High HIF-1a gene expression was associated with worse response to this treatment.

Moreover the relatively good prognosis of patients with HPV+ OPSCC is also reduced when there is high expression of HIF-1a. The major drivers behind this reduced prognosis and strategies to normalize oxygenation should be investigated.

Hypoxia and HIF-targeted therapies: when will the time be right?

As immunotherapy is gaining popularity and has proven effect on patient outcome, one cannot help but wonder why hypoxic-modification of treatment is not already a part of clinical practice as well: There is class IA evidence that hypoxic-modification of treatment is beneficial for patient outcomes in HNSCC.[20] In 2011, the European Medicines Agency has approved an orphan-drug status that allows nimorazole to be used during radiotherapy for head and neck cancer.[21] In addition, a validated hypoxia gene expression classifier has been described that discerns responders from non-responders to nimorazole addition.[22] In Denmark, nimorazole is routinely administered to patients as a radiosensitizer. Interestingly, efforts to perform an international phase III study of nimorazole could not be completed because of accrual issues.[23]

Still, there are ongoing trials to investigate the effects of nimorazole in HNSCC.[24] This includes the phase III NIMRAD study that investigates nimorazole in patients with an indication for, but who are not suitable for treatment with concomitant cisplatin or cetuximab. [25] Another study is NCT01880359, a phase III trial on the effectiveness of nimorazole in combination with cisplatinum-based chemotherapy in HPV- LSCC, OPSCC and hypopharyngeal SCC. Several other hypoxia-activated prodrugs (HAPs) were promising in phase I and II trials, but no phase III trials were undertaken.[15,24,26] As power issues are important in such trials (i.e. the number of patients needed to include in a trial), it is possible

to create a step-wise inclusion for these studies where the hypoxic status is assessed prior to randomization to reduce the number needed to include patients who are likely to benefit most from these HAPs.[26] Excluding patients with well-oxygenated tumors makes sense as hypoxia is highly variable between tumors and improving oxygenation of well-oxygenated tumors will negatively bias the results of phase III trials.

In addition, as this research shows the co-existence of various forms of hypoxia, it also shows that different methods to detect hypoxia (endogenous and exogenous markers, hypoxia gene expression classifiers and imaging modalities such as hypoxia PET) should be seen as complementary rather than superior or inferior. Perhaps response prediction for each hypoxia targeted treatment will require a different imaging or tissue based biomarker.

Alternative approaches to improve tumor hypoxia include treatments to improve vascularity in tumors, such as VEGF inhibitors as described above. And while the benefit of accelerated radiotherapy (AR) with carbogen and nicotinamide (ARCON) was modest in a phase III trial of laryngeal squamous cell carcinoma patients, there were subgroups that had significant benefit of this therapy, such as patients with pre-treatment anemia, patients with a perinecrotic staining pattern of the EMH CA-IX or patients selected with a gene classifier.[27–29]

Because of the poor prognosis of patients with HNSCC, there is much to gain from novel therapies targeting hypoxia and/or HIF. While there seems to be a focus in the field on immune therapy, hypoxia targeted therapies may be a useful adjunct in patients with hypoxic and should not be forgotten. There is a need for phase III trials to investigate promising hypoxia directed therapies (HAPs, VEGF inhibitors, ARCON) particularly in patients with hypoxic tumors.

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Chapter 9

Nederlandse samenvatting

Lijst van publicaties

Dankwoord

Over de auteur

Nederlandse samenvatting

Hoofd-halskanker

Hoofd-halskanker is de zesde meest voorkomende vorm van kanker in Nederland. Deze term is een overkoepelende term voor onder andere kanker van de mondholte, de keel en het strottenhoofd (inclusief de stembanden). In de meeste gevallen betreft het een plaveiselcelcarcinoom. Deze vorm van kanker ontstaat door een woekering van kwaadaardig geworden (plaveisel-) cellen die de slijmvliezen bekleden. In de klassieke vorm wordt het ontstaan van een hoofd-hals plaveiselcelcarcinoom (HHPCC) geassocieerd met roken en alcoholgebruik. Sinds enige jaren is binnen deze ziekte een subgroep van jongere en niet-rokende patiënten ontdekt, bij wie HHPCC ontstaat door besmetting met het humaan papillomavirus (HPV). Dit is hetzelfde virus dat bijvoorbeeld baarmoederhalskanker veroorzaakt. Patiënten met een HPV-gerelateerd HHPCC worden vaker met een uitgebreidere tumor gediagnosticeerd, maar hebben interessant genoeg wel een betere prognose dan patiënten met niet-HPV gerelateerd HHPCC. Deze tumoren reageren namelijk beter op bestraling (radiotherapie) met of zonder chemotherapie.

De biologische eigenschappen van een tumor hebben dus een direct effect op de prognose van een patiënt, onafhankelijk van de uitgebreidheid van de ziekte. Om die reden is HPV-status in de meest recente classificatie (TNM8) opgenomen als een aparte entiteit. Zo kan een grote tumor in bepaalde gevallen classificeren als stadium I ziekte (het laagste/gunstigste stadium) als deze HPV-gerelateerd is en stadium IV (het hoogste stadium) als deze niet HPV-gerelateerd is. De zogeheten tumorbiologie is momenteel een belangrijk onderwerp van onderzoek. De kennis hierover kan gebruikt worden om de overlevingskans van een patiënt te voorspellen (prognostisch), maar ook om de succeskans van een behandeling te voorspellen (predictief of voorspellend). Hopelijk kan hierdoor in de toekomst voor elke patiënt op basis van patiënt- en tumorkenmerken de behandeling worden geselecteerd die hem of haar de beste kans op genezing geeft.

De tumor als micro-omgeving

Tumoren bestaan niet slechts uit een klompje identieke kwaadaardige cellen zijn, maar uit tal van verschillende cellen. **Hoofdstuk 1, Figuur 3** illustreert dit verschijnsel. Tumorcellen leven in een samenspel met tal van andere soorten cellen, waaronder bloedvaten, immuuncellen en steuncellen. Dit samenspel noemen we de tumor micro-omgeving. Dit is van belang, omdat alle onderdelen binnen deze tumor micro-omgeving potentiële aangrijpingspunten zijn voor een behandeling. Een voorbeeld hiervan is immunotherapie, waarbij het eigen immuunsysteem van de patiënt wordt geactiveerd om tumoren te remmen

of te vernietigen. Deze behandeling blijkt voor veel kankersoorten succesvol te zijn en wordt daarom in de laatste jaren steeds vaker en in meer verschillende soorten kanker toegepast.

Hypoxie

Een andere factor in de tumor micro-omgeving is hypoxie (zuurstoftekort). Omdat een tumor vaak sneller groeit dan zijn eigen bloedvatvoorziening kan bijbenen, ontstaan er in een tumor regelmatig gebieden met hypoxie. Hypoxie kan acuut zijn, zoals wanneer er (tijdelijk) een bloedvat dichtgedrukt of afgesloten wordt door tumorcellen of een bloedpropje. Hypoxie kan ook chronisch zijn wanneer deze belemmering meer geleidelijk optreedt. Dit kan ontstaan wanneer tumorcellen zich sneller vermenigvuldigen dan de bloedvaten. In verhouding zijn er dan te weinig bloedvaten en ontstaat er dus een tekort aan bloedtoevoer. Het kan ook ontstaan doordat er juist te veel bloedvaten door een tumor lopen. Het bloed kan dan niet efficiënt door de tumor stromen. Deze beide verschijnselen worden getoond in **Hoofdstuk 1, Figuur 5**. In de praktijk komen beide vormen van hypoxie vaak gelijktijdig voor.

Er zijn verschillende methoden beschikbaar om hypoxie aan te tonen. In dit proefschrift wordt gebruik gemaakt van biomarkers. Een biomarker is een hulpmiddel om een bepaalde toestand of eigenschap binnen (tumor-)weefsel aan te tonen. Het is vaak een stof of eiwit waarvan de aanwezigheid correleert met de toestand of eigenschap die je wilt onderzoeken. Met andere woorden: als er een hoge concentratie van een biomarker wordt gemeten verwacht je dat het weefselkenmerk aanwezig is. Is er een lage concentratie of geen biomarker aanwezig, verwacht je dat het weefselkenmerk niet aanwezig is. Er bestaan verschillende biomarkers voor hypoxie. In de onderzoeken in dit proefschrift werd onder andere gebruik gemaakt van de exogene biomarker pimonidazol. Deze biomarker is exogeen omdat deze met een infuus moet worden toegediend aan de patiënt voordat er een biopt ('weefselhapje') wordt uitgenomen. De biomarker stroomt via de bloedvaten naar de tumor toe en wordt gebonden in de hypoxische cellen. Als het biopt is genomen kan de aanwezigheid van pimonidazol worden aangetoond middels immunohistochemie (een weefselkleuringstechniek). Naast pimonidazol werd gebruik gemaakt van het endogene biomarker-eiwit Hypoxia-Inducible Factor 1-alpha (*door hypoxie induceerbare factor 1-*alpha, ofwel HIF-1a). Deze biomarker is endogeen omdat deze van nature in hypoxische cellen aanwezig is: dit eiwit wordt in alle cellen continu aangemaakt, maar in cellen met voldoende zuurstof ook direct weer afgebroken. In hypoxische cellen kan dit afbraakproces niet plaatsvinden en daardoor neemt de hoeveelheid HIF-1a toe (Zie ook **Hoofdstuk 1, Figuur 6**). Voor een cel is dit nuttig, omdat HIF-1a aanzet tot productie van eiwitten die de cel helpen te overleven in hypoxische situaties. Ook de aanwezigheid van HIF-1a kan worden aangetoond met immunohistochemie.

Zoals eerder aangegeven kunnen biomarkers prognostisch zijn (om bijvoorbeeld de overlevingskans van een patiënt te berekenen), maar ook predictief (om de succeskans van een behandeling te berekenen en zo te helpen beslissen welke behandeling het beste gegeven kan worden). Dit werkt voor hypoxie als biomarker net zo: het onderscheiden van normoxische en hypoxische tumoren kan prognostisch gebruikt worden om patiënten te identificeren met een betere of slechtere prognose. Echter omdat er behandelingen in ontwikkeling die zich specifiek richten op hypoxische cellen kunnen hypoxiemarkers ook predictief zijn: het identificeren van hypoxische tumoren kan helpen om patiënten te selecteren voor hypoxie-gerichte behandelingen.

Dit proefschrift

Een van de doelen van dit proefschrift was om meer inzicht te krijgen in de relatie van tumorhypoxie met andere factoren binnen de tumor micro-omgeving. Hiernaast werd gekeken naar de effecten van tumorhypoxie op patiëntuitkomsten. Deze beide onderdelen moeten er uiteindelijk aan bijdragen erachter te komen of hypoxie-gerichte behandelingen plaats hebben in de behandeling van patiënten met HHPCC.

Het onderzoek beschreven in **Hoofdstuk 2** vergeleek de hypoxie biomarkers pimonidazol en HIF-1a in 44 patiënten met strottenhoofdkanker (larynxcarcinoom). Deze patiënten deden mee aan een gerandomiseerde studie waar het lot had bepaald of patiënten behandeld zouden worden met gewone bestraling of ARCON. ARCON is een hypoxie-gerichte behandeling. Het bestaat uit een combinatie van bestraling, een vaatverwijdend medicijn (nicotinamide) en het inademen van een zuurstof gasmengsel vlak voor en tijdens de bestraling (carbogen, een mix van 2% CO_2 en 98% zuurstof). Het doel van ARCON is om tijdens de bestraling de zuurstofconcentratie in het bloed te verhogen om zo tumorhypoxie te verminderen of op te heffen. Voor dit onderzoek werden met immunohistochemie gekleurde weefselcoupes ingescand en werd digitaal de aanwezigheid van HIF-1a en pimonidazol gemeten. Normaal gesproken gebeurt dit door handmatige (visuele) beoordeling door een patholoog. Met deze computerbepaling kon niet alleen het percentage cellen met HIF-1a of pimonidazol gemeten worden, maar kon ook ruimtelijk binnen het weefsel gekeken worden in welke gebieden aankleuring plaatsvond en of er overlap was tussen de twee kleuringen. De belangrijkste bevinding was dat het aantal gedetecteerde hypoxische gebieden goed overeenkwam tussen HIF-1a en pimonidazol, maar dat de gebieden van aankleuring niet altijd overlapten. Wij concludeerden dat beide biomarkers geschikt zijn om hypoxie in weefsel te bepalen, maar dat beide biomarkers andere vormen van hypoxie detecteren en elkaar dus aanvullen.

In **Hoofdstuk 3** hebben we de relatie onderzocht tussen hypoxie en twee andere factoren in de tumor micro-omgeving: de aanwezigheid van het eiwit PD-L1 (een eiwit van belang voor immunotherapie) en het aantal immuuncellen dat zich in een tumor bevinden. Dit werd

onderzocht in een cohort van 274 patiënten met keelkanker. Wij vonden dat hypoxische tumoren vaker minder immuuncellen bevatten. Tumoren met veel PD-L1 expressie bevatten vaak juist meer immuuncellen. Er werd geen relatie gevonden tussen hypoxie en PD-L1 expressie. De aanwezigheid van meer immuuncellen en niet-hypoxische tumoren waren beide onafhankelijke voorspellers van een betere overleving. Deze studie onderschrijft dat er interactie is tussen de verschillende componenten van de tumor micro-omgeving en dat deze factoren onafhankelijk samenhangen met de prognose voor patiënten.

In **Hoofdstuk 4** werden de kenmerken hypoxie, immuuncel-aantallen en celproliferatie (de snelheid waarmee cellen zich vermenigvuldigen) onderzocht en vergeleken met de beeldvormingstechniek diffusie-gewogen *Magnetic Resonance Imaging,* of MRI. MRI wordt vrij vertaald als 'beeldvorming door magnetische resonantie' en is een scan waarbij de beelden worden gemaakt op basis van magnetische principes. Met diffusie-gewogen MRI wordt bekeken in welke gebieden in een tumor watermoleculen zich vrij kunnen bewegen. In gebieden waar water vrij kan stromen is er geen diffusierestrictie en in gebieden waar er veel blokkades zijn is er veel diffusierestrictie. Onze hypothese was dat hypoxie leidt tot celdood en er minder diffusierestrictie zou zijn. Daarnaast verwachtten wij dat een hoge celproliferatie en een hoger aantal (kleine) immuuncellen beide zouden leiden tot hogere celdichtheid en daardoor meer diffusierestrictie. In de studie werden gegevens van overlappende patiënten van twee andere, reeds verrichte studies gebruikt. Dit resulteerde in data van 20 patiënten. De verwachte hypothesen over immuuncel aantallen en proliferatie werden beide gevonden. Wat betreft hypoxie werd er wel een correlatie gevonden in de verwachte richting, echter de correlatie was niet zo sterk dat dit ook statistisch significant was.

De studies hierna gingen over de effecten van hypoxie op patiëntuitkomsten. In **Hoofdstuk 5** verrichtten wij een literatuuronderzoek naar verschillende endogene biomarkers voor hypoxie (EMH). Hier identificeerden wij 40 verschillende studies naar het effect van expressie van EMHs op patiëntuitkomsten in HHPCC. Er werden verschillende EMHs beschreven, waarbij HIF-1a wel de vaakst beschreven EMH is. Opvallend was dat er veel verschillende manieren gehanteerd werden om de expressie van EMHs te beoordelen. Daarnaast werden er veel verschillende afkapwaarden voor positiviteit werden gebruikt. In de meeste studies werd expressie van EMHs geassocieerd met slechtere patiëntuitkomsten. Daarnaast werd in veel studies EMH positiviteit ook gezien in tumoren in een hoger stadium of een slechtere differentiatiegraad (een maat voor hoe afwijkend of kwaadaardig de cellen eruitzien onder de microscoop). Het viel ook op dat expressie van EMHs zowel bij een chirurgische behandeling als bij een orgaansparende behandeling (radiotherapie) een negatief effect op de uitkomst had. Dus: tumorhypoxie wordt geassocieerd met een slechtere prognose, ook als de hypoxische tumor in zijn geheel verwijderd wordt. Dit geeft aan dat voor chirurgische en niet-chirurgische patiëntengroepen behandeling gericht op de hypoxie óf gericht op HIF-1a van belang kan zijn.

In **Hoofdstuk 6** hebben wij in ons eigen cohort van 274 keelkankerpatiënten het effect op overleving onderzocht van HIF-1a expressie en twee gerelateerde EMHs: CA-IX en GLUT-1. HIF-1a was in deze studie een sterkere voorspeller van de prognose dan CA-IX en GLUT-1. Daarnaast werd ook gekeken naar het effect van HIF-1a expressie in relatie tot HPV status. In de regel hebben patiënten met een HPV-gerelateerde tumor een betere prognose dan patiënten met een niet-HPV gerelateerde tumor. Wij vonden dat de prognose van patiënten met een HPV-gerelateerde tumor die ook HIF-1a expressie toonde vergelijkbaar was met (of: even slecht als) patiënten die een niet-HPV gerelateerde tumor hadden. Dit is met name relevant omdat er momenteel studies worden verricht om patiënten met HPV-gerelateerde tumoren een minder zware behandeling te geven. Dit wordt de-escalatie van de behandeling genoemd. Volgens ons onderzoek zijn patiënten met een HPV-gerelateerde maar hypoxische tumor dus geen goede kandidaat voor de-escalatie.

Om ten slotte nog verdere vergelijking te maken tussen HHPCCs van verschillende gebieden werden in **Hoofdstuk 7** patiënten met verschillende tumoren vergeleken. Dit waren 391 patiënten met mondkanker, 248 patiënten met strottenhoofdkanker en 301 patiënten met keelkanker. Een belangrijk verschil tussen mensen met mondkanker en mensen met keel- of strottenhoofdkanker is dat deze meestal operatief behandeld worden. Mensen met keel- of strottenhoofdkanker worden vaker met bestraling met of zonder chemotherapie en minder vaak met een operatie. Wij vonden dat HIF-1a expressie veel vaker voorkwam in mondkanker dan in de andere twee groepen. Interessant genoeg was bij patiënten met mondkanker de aanwezigheid van HIF-1a expressie een voorspeller voor een bétere prognose, in tegenstelling tot bij patiënten met keel- of strottenhoofdkanker. Dit effect kon niet verklaard worden door de verschillen in behandeling (operatief versus bestraling). Een mogelijke verklaring is dat HIF-1a expressie bij mondkanker tot stand komt door andere processen dan door hypoxie. Patiënten met mondkanker hebben dus mogelijk geen baat bij behandeling gericht tegen HIF-1a of hypoxie.

Conclusie

Hypoxie en HIF-1a expressie lijken relevant te zijn in patiëntengroepen met mond- keel- of strottenhoofdkanker, of deze nu chirurgisch of niet-chirurgisch behandeld worden. Hypoxie is dus een potentieel aangrijpingspunt voor behandeling bij patiënten met HHPCC, net zoals bijvoorbeeld immuuntherapie dit nu is. Verscheidene hypoxie-gerichte behandeling worden al in onderzoeksverband toegepast bij patiënten met HHPCC, maar maken nog geen deel uit van de 'gewone' behandeling.

Er zijn wezenlijke verschillen tussen verschillende tumoren binnen hetzelfde gebied. Om voor elke patiënt de beste behandeling te selecteren is het dus belangrijk om meer inzicht te hebben in de tumorbiologie middels scans en/of biomarkers. Op basis van deze kenmerken zou uiteindelijk de beste behandeling voor elke specifieke tumor gekozen

moeten worden. Ook een hypoxie-gerichte behandeling lijkt opportuun voor patiënten met HHPCC. Er zijn echter verschillende soorten hypoxie gerichte behandelingen mogelijk en wellicht is voor elke behandeling een andere biomarker het best voorspellend. De specifieke rol van HIF-1a als een voorspellende biomarker zou in elke hypoxiebehandeling apart onderzocht moeten worden. Daarnaast zou HIF-1a zelf ook een aangrijpingspunt voor behandeling kunnen zijn. Momenteel worden in laboratoriumonderzoeken op losse tumorcellen medicijnen onderzocht die HIF-1a remmen, met als doel de cellen gevoeliger te maken voor behandeling.

De immense recente populariteit van immunotherapie in kankerpatiënten illustreert dat er ruimte is voor nieuwe behandelingen die aangrijpen op andere aspecten van de tumor en de tumor micro-omgeving naast de klassieke behandelvormen van operatie, bestraling of chemotherapie. De relatie tussen HIF-1a en het immunotherapie gerelateerde eiwit PD-L1 illustreert dat bijvoorbeeld een combinatie van immunotherapie en hypoxie-gerichte behandeling mogelijk leidt tot een betere prognose. De hoofdstukken van dit proefschrift geven aan dat er mogelijkheden liggen voor hypoxie- en HIF-1a gerichte behandelingen in het behandelarmentarium voor patiënten met HHPCC.

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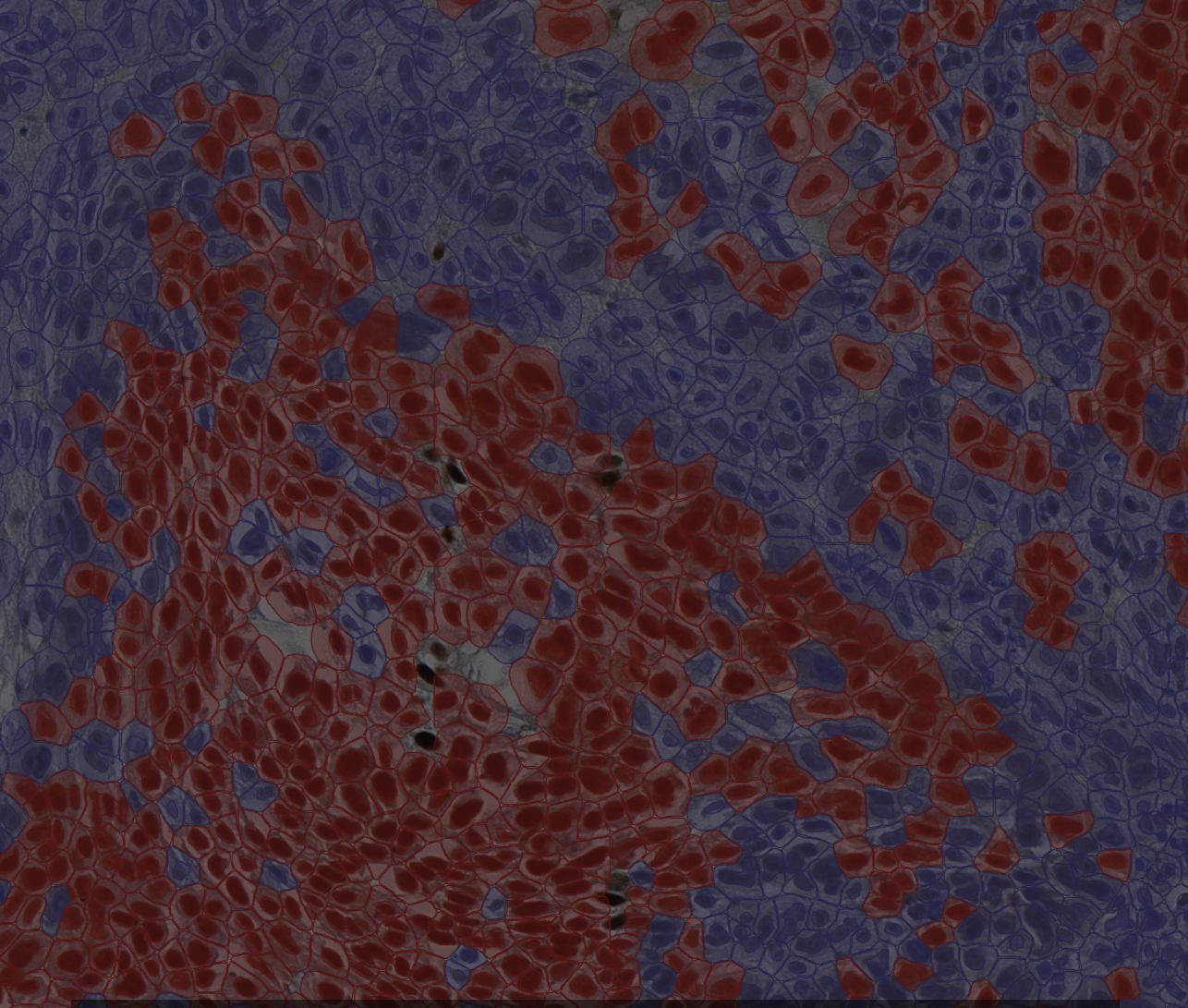
Over de auteur

Justin Egidius Swartz werd op 6 september 1989 als oudste zoon geboren te Hoorn in een gezin met Indische *roots*. Hij rondde het Gymnasium af aan het Martinus College in Grootebroek, waarna hij in 2007 begon aan zijn studie Geneeskunde aan de Universiteit Utrecht. Hij volgde onder andere coschappen aan de universiteit van Kuala Lumpur, Maleisië. Tijdens zijn studie werkte hij als hoofddocent Wiskunde A bij de Stichting Studiebegeleiding in Leiden, waar hij zijn echtgenote Emma heeft ontmoet. Zijn wetenschappelijke carrière begon hij in de virologie onder leiding van dr. A.M.J. Wensing, resulterend in een publicatie en een presentatie op een internationaal congres in Rome.

Kort hierna ontdekte hij zijn interesse in de keel-neus-oorheelkunde en de hoofd-halsoncologie. Hij startte zijn promotieonderzoek naar hypoxie in hoofd-halstumoren in het UMC Utrecht in 2014. Tijdens deze periode maakte hij ook verschillende wetenschappelijke uitstapjes naar andere onderwerpen binnen de KNO. In 2015 won hij de 1e prijs beste presentatie op de NWHHT Jonge Onderzoekersdag. In 2016 startte hij zijn opleiding tot KNO-arts (opleider drs. I. Ligtenberg), met een differentiatie in de hoofd-halsoncologie en weke delenchirurgie in het UMC Utrecht en het Antoni van Leeuwenhoek Ziekenhuis.

Na zijn opleiding te hebben afgerond volgde hij een door de European Academy of Facial Plastic Surgery (EAFPS) geaccrediteerd fellowship Aangezichtschirurgie in het Ziekenhuis Gelderse Vallei te Ede en het Radboudumc te Nijmegen bij dr. W. Boek en dr. K.J.A.O. Ingels. Hij werkt momenteel als KNO-arts in het Jeroen Bosch Ziekenhuis en zal per september 2023 toetreden tot de vakgroep KNO van het Diakonessenhuis te Utrecht.

Hij is op 8 juli 2016 getrouwd met Emma Swartz – den Hollander. Op 3 oktober 2019 kregen zij Abel Judah Swartz en Benja Sem Swartz. Zij wonen momenteel in Houten.



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Awareness increases that tumors are not a homogenous collection of cells but instead form a micro-environment together with non-cancer cells. Novel anti-cancer therapies such as immunotherapy target this micro-environment rather than tumor cells directly. Hypoxia (low oxygen levels within a tumor) often exists within solid tumors and is another potential treatment target in the tumor micro-environment. This research describes the occurrence and effects of tumor hypoxia in patients with head and neck squamous cell carcinoma. Hypoxia was encountered frequently and was in most cases associated with worse prognosis. In addition, the presence of hypoxia affected other tumor micro-environment factors as well. The studies in this dissertation highlight the potential role of hypoxia-targeted therapies. These therapies should be re-explored in the modern treatment paradigm.

– Justin E. Swartz, 2023

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