Validation of PET imaging for non-invasive characterization of head and neck tumors

Esther G.C. Troost
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Esther Gera Cornelia Troost
18FLT-PET-CT image of a patient with a cT4N2c oral cavity tumor. The PET signal is overlaid by a pseudo-colored immunohistochemical image of a frozen tumor biopsy showing proliferating tumor cells (red), hypoxic tumor cells (green) and vessels (blue). Courtesy of Jasper Lok.
Validation of PET imaging for non-invasive characterization of head and neck tumors

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Voor mijn ouders
Voor Aswin
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<td>ARCON</td>
<td>accelerated radiotherapy with carbogen and nicotinamide</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumine</td>
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<tr>
<td>BOLD</td>
<td>blood oxygen level dependence</td>
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<tr>
<td>BrdUrd</td>
<td>bromodeoxyuridine</td>
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<td>BRU</td>
<td>2-nitroimidazole agent</td>
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<td>11C</td>
<td>carbon-11</td>
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<td>11C-MET</td>
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<td>CA-IX</td>
<td>carbonic anhydrase IX</td>
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<td>CCD</td>
<td>charge-coupled device</td>
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<td>CCI-103F</td>
<td>hexafluoromisonidazole</td>
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<tr>
<td>CI</td>
<td>confidence interval</td>
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<tr>
<td>CT</td>
<td>computed tomography</td>
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<td>CTV</td>
<td>clinical target volume</td>
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<td>$^{60}$Cu</td>
<td>copper-60</td>
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<td>$^{60}$Cu-ATSM</td>
<td>$^{60}$Cu(H)-diacetyl-bis(N^4-methylthiosemicarbazone)</td>
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<td>DAB</td>
<td>diaminobenzidine</td>
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<td>DCE-CT</td>
<td>dynamic contrast enhanced CT</td>
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<td>DCE-MRI</td>
<td>dynamic contrast enhanced MRI</td>
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<tr>
<td>DSC</td>
<td>Dice Similarity Coefficient</td>
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<tr>
<td>EF5</td>
<td>2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl)-acetamide</td>
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<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>f</td>
<td>female</td>
</tr>
<tr>
<td>9F1</td>
<td>rat monoclonal to mouse endothelium</td>
</tr>
<tr>
<td>18F</td>
<td>fluorine-18</td>
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<td>18F-AZA</td>
<td>18F-fluoroazomycin arabinoside</td>
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<td>18F-EFTNIM</td>
<td>18F-fluoroerythronitromidazole</td>
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<td>18F-2-(2-nitroimidazol-1-yl)-N-(3,3,3-trifluoropropyl)-acetamide</td>
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<td>18F-fluorodeoxyglucose</td>
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<td>18F-FET</td>
<td>0-2-18F-fluoroethyl-L-tyrosine</td>
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<td>3’-deoxy-3’-18F-fluorothymidine</td>
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<td>18F-FMISO</td>
<td>18F-fluoromisonidazole</td>
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<td>fCA-IX</td>
<td>fraction positive for CA-IX</td>
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<td>Fig</td>
<td>figure</td>
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<tr>
<td>FN</td>
<td>false negative</td>
</tr>
<tr>
<td>FP</td>
<td>false positive</td>
</tr>
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<td>fPMO</td>
<td>fraction positive for pimonidazole</td>
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<tr>
<td>G250</td>
<td>antibody against CA-IX</td>
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<tr>
<td>GC</td>
<td>germinal center</td>
</tr>
<tr>
<td>GLUT-1</td>
<td>glucose transporter 1</td>
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<tr>
<td>GTV</td>
<td>gross tumor volume</td>
</tr>
<tr>
<td>GTV$_{50%}$</td>
<td>GTV based on 50% isocontour</td>
</tr>
<tr>
<td>GTV$_{80%}$</td>
<td>GTV based on 80% isocontour</td>
</tr>
<tr>
<td>GTV$_{SBR}$</td>
<td>GTV based on SBR</td>
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<tr>
<td>Gy</td>
<td>Gray [J/kg$^{-1}$]</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
</tr>
<tr>
<td>HF</td>
<td>hypoxic fraction</td>
</tr>
<tr>
<td>HIF-1alpha</td>
<td>hypoxia-induced factor 1 alpha</td>
</tr>
<tr>
<td>HPV</td>
<td>human papillomavirus</td>
</tr>
<tr>
<td>111In</td>
<td>indium-111</td>
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<tr>
<td>IdUrd</td>
<td>iododeoxyuridine</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
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<tr>
<td>IMRT</td>
<td>intensity-modulated radiation therapy</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>ITLC</td>
<td>instant thin-layer chromatography</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>Ki-67</td>
<td>endogenous proliferation marker, protein encoded by MKI67 gene</td>
</tr>
<tr>
<td>kV</td>
<td>kilo Volt</td>
</tr>
<tr>
<td>LI</td>
<td>labeling index</td>
</tr>
<tr>
<td>LN</td>
<td>lymph node</td>
</tr>
<tr>
<td>LT</td>
<td>lymphoid tissue</td>
</tr>
<tr>
<td>M</td>
<td>molarity $[\text{mol-L}^{-1}]$</td>
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<tr>
<td>m</td>
<td>male</td>
</tr>
<tr>
<td>mAB</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>mAs</td>
<td>milliAmpere second</td>
</tr>
<tr>
<td>MBq</td>
<td>mega Becquerel</td>
</tr>
<tr>
<td>MET</td>
<td>metastases</td>
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<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>MRND</td>
<td>modified radical neck dissection</td>
</tr>
<tr>
<td>MRS</td>
<td>magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>n.a.</td>
<td>not available</td>
</tr>
<tr>
<td>n.p.</td>
<td>not performed</td>
</tr>
<tr>
<td>n.r.</td>
<td>not representative</td>
</tr>
<tr>
<td>NS</td>
<td>node sampling</td>
</tr>
<tr>
<td>n.s.</td>
<td>not significant</td>
</tr>
<tr>
<td>$^{15}$O</td>
<td>oxygen-15</td>
</tr>
<tr>
<td>$^{15}$O-H$_2$O</td>
<td>$^{15}$O-labeled water</td>
</tr>
<tr>
<td>OR</td>
<td>odds ratio</td>
</tr>
<tr>
<td>OSEM</td>
<td>ordered subset expectation maximization</td>
</tr>
<tr>
<td>PAD</td>
<td>primary antibody diluent</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
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<tr>
<td>PET</td>
<td>positron emission tomography</td>
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<tr>
<td>PIMO</td>
<td>pimonidazole</td>
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<tr>
<td>pimonidazole</td>
<td>1-[(2-hydroxy-3-piperidinyl)propyl]-2-nitroimidazole hydrochloride</td>
</tr>
<tr>
<td>pO$_2$</td>
<td>arterial partial pressure of oxygen</td>
</tr>
<tr>
<td>PTV</td>
<td>planning target volume</td>
</tr>
<tr>
<td>PPI</td>
<td>phosphor imaging system</td>
</tr>
<tr>
<td>RCHT</td>
<td>radiotherapy + chemotherapy</td>
</tr>
<tr>
<td>RGB</td>
<td>red-green-blue</td>
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<tr>
<td>RND</td>
<td>radical neck dissection</td>
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<tr>
<td>RTL</td>
<td>relative-threshold level</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SBR</td>
<td>signal-to-background ratio</td>
</tr>
<tr>
<td>SIB</td>
<td>simultaneous integrated boost</td>
</tr>
<tr>
<td>SND</td>
<td>selective neck dissection</td>
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<tr>
<td>SPECT</td>
<td>single photon emission computed tomography</td>
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<tr>
<td>SPET</td>
<td>single photon emission tomography</td>
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<tr>
<td>SUV</td>
<td>standardized uptake value</td>
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<td>SUV$_{\text{max}}$</td>
<td>maximum SUV</td>
</tr>
<tr>
<td>SUV$_{\text{mean}}$</td>
<td>mean SUV</td>
</tr>
<tr>
<td>$^{99m}$Tc</td>
<td>technecium-99m</td>
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<tr>
<td>TE</td>
<td>tumor excision</td>
</tr>
<tr>
<td>TK-1</td>
<td>thymidine kinase 1</td>
</tr>
<tr>
<td>TLE</td>
<td>total laryngectomy</td>
</tr>
<tr>
<td>TN</td>
<td>true negative</td>
</tr>
<tr>
<td>TP</td>
<td>true positive</td>
</tr>
<tr>
<td>US</td>
<td>ultrasound</td>
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Chapter 1

Introduction and outline
1.1 Treatment of head and neck carcinomas

Worldwide, approximately 850,000 patients annually present with squamous cell carcinomas of the head and neck, and in the Netherlands the incidence is 2,400 (www.oncoline.nl). As such, head and neck cancer is ranked as the fifth most common malignancy in men and eighth in women. In western countries, patients typically have a history of alcohol and nicotine abuse, although increasing evidence on the role of the human papilloma virus emerges in relatively young non-smoking oropharyngeal cancer patients. In Asia and parts of Africa, infection with the Epstein-Barr virus and betel quid chewing play an important role in the development of nasopharyngeal and oral cancer, respectively. As patients’ complaints are often rather vague varying from throat ache and difficulties with swallowing to otalgia, they often present with advanced stage disease: large, bulky primary tumors and frequently uni- or bilateral cervical lymph node metastases. Generally, head and neck cancer first poses a loco-regional problem, however, the rate of distant (pulmonary) metastases depends on the primary tumor site and stage. Therefore, multi-modality treatment primarily focuses on loco-regional control by surgical resection of the tumor and cervical lymph node levels, radiotherapy of the tumor and (potentially) affected lymph nodes, chemotherapy for enhanced radiation efficacy, or a combination of these. Due to an increasing preference for organ preservation strategies, radiotherapy is the prime modality in most cases.

1.2 Resistance mechanisms in head and neck tumors

Three major tumor characteristics are known that adversely influence radiotherapy outcome and prognosis: tumor cell hypoxia, clonogenic proliferation and intrinsic radiosensitivity.

Intrinsic radiosensitivity of squamous cell carcinomas is generally classified as “intermediate” albeit with broad intertumor variations. Factors such as DNA single- and double-strand repair capacity, and apoptotic potential influence resistance to radiotherapy.

Accelerated tumor cell proliferation is induced as response to cell loss during radiotherapy. It is affected by several factors, such as differentiation of the tumor, cell-cycle gene regulation, and microenvironmental factors, including oxygen and nutrient availability. Retrospective analyses of radiation dose and time relations in head and neck cancer have revealed a decrease in local tumor control with increasing overall treatment time.

Hypoxia appears to be present in the majority of solid human tumors, including squamous cell carcinomas of the head and neck. Traditionally, two major forms of hypoxia are recognized: “chronic” or “diffusion-limited”, and “acute” or “perfusion-limited”. Tumors are vascularized by a chaotic network of blood vessels with changing
Introduction and outline

vessel diameters, leaky vessel walls and arterio-venous shunts. Chronic hypoxia is caused by an exceeding of the maximum diffusion capacity between the tumor cells and the supplying blood vessel leading to a depletion of nutrients and oxygen. The underlying mechanism for acute hypoxia is the transient opening and closure of malformed blood vessels resulting in fluctuations of blood perfusion to tumor regions and temporary changes in oxygen tensions. Often the two types of hypoxia co-exist, while dominance of one type or the other may vary over time. There is ample evidence that the tumor oxygenation status predicts the prognosis of head and neck and cervical cancer patients undergoing radiotherapy. Hypoxia-induced genome and proteome changes may promote tumor progression via various mechanisms. These alterations enable cells to overcome oxygen and nutrient deprivation, help cells to escape from hostile environment and favor unrestricted growth. Cells thus change to a clinically more aggressive phenotype with increased potential for local invasive growth, perifocal tumor cell spreading and formation of regional and distant metastases, all negatively affecting prognosis.

1.3 Treatment modifications counteracting tumor cell hypoxia and accelerated proliferation

Shortening of the overall treatment time is an established method to counteract tumor cell repopulation. “Accelerated radiotherapy” reduces the overall treatment time by delivering more than one fraction per day, and/or by continuing treatment during the weekends. With acceptable acute toxicity, the overall treatment time can be reduced up to two weeks whilst maintaining the same total radiation dose. Several randomized trials and one meta-analysis have demonstrated the effectiveness of this approach.

Numerous strategies to overcome tumor cell hypoxia have been clinically tested. A large meta-analysis including 86 randomized clinical trials on various primary tumor sites, including 31 trials on head and neck cancer, has shown that radiotherapy with oxygenation modifying treatment resulted in improved loco-regional control [odds ratio (OR) 0.77; 95% confidence interval (CI), 0.71 to 0.86] and survival (OR 0.87; 95% CI, 0.80 to 0.95) compared to radiotherapy alone. This study included trials with hyperbaric oxygen, normobaric oxygen, carbogen, hypoxic cell radiosensitizers, or a combination of hyperbaric oxygen with hypoxic sensitizer.

A large phase II trial on a patient cohort with advanced stage head and neck cancer was conducted applying Accelerated Radiotherapy with CarbOgen breathing and Nicotinamide (ARCON). In this clinical study, accelerated radiotherapy was given to counteract accelerated tumor cell repopulation occurring during the course of treatment. Patients breathed carbogen (98% O₂ and 2% CO₂) in order to raise the arterial partial pressure of oxygen (pO₂) and thus decrease diffusion-limited (chronic) hypoxia. The third component, nicotinamide, was orally administered in order to
decrease perfusion-limited (acute) hypoxia by preventing intermittent vascular shutdown. Nicotinamide probably also influences metabolic processes, including oxidative metabolism and glycolysis, that shift the oxygen consumption rate and acid-base balance, and secondarily affect tumor blood flow and pO2. In this study, this approach resulted in high local and regional control rates, especially for advanced stage laryngeal and oropharyngeal cancer (actuarial 3-year control rates for the primary tumor: 80% and 88%, and for the cervical lymph nodes: 95% and 85%, respectively) 37. Therefore, a large phase III clinical trial addressing the value of ARCON including 345 laryngeal cancer patients was initiated and recently completed. Results are expected to be communicated in the year 2010.

1.4 Selection of patients

Although the long-term figures regarding therapeutic outcome after treatment modification are encouraging, not all patients benefit. Patients may present with tumors that are barely hypoxic or do not develop accelerated repopulation during irradiation. Intensified treatment is associated with increased (acute) toxicity: prolonged radiation induced mucositis of increased severity, and longer dependence on temporary feeding by nasogastric tube or percutaneous gastrostomy 37. Therefore, it is mandatory to select those patients who are most likely to profit from treatment modification prior to treatment and possibly also to adjust the treatment in an early phase in case of insufficient response. This requires predictive assays that reliably assess tumor cell hypoxia and proliferation prior to and during treatment.

The polarographic needle electrode (Eppendorf™) directly measures pO2 in accessible primary tumors and cervical lymph node metastases 24,39,40. The disadvantages of this method include: insufficient coverage of the entire tumor or lymph node, and limitations regarding repetitive readings when the procedure must be performed under general anesthesia. The most important limitations, however, are that this method cannot distinguish necrosis with very low pO2 readings from severe hypoxia in viable tumor areas, and that it does not provide information on the relation of hypoxia to the histological architecture and the microenvironment of the tumor.

Tumor cell hypoxia and proliferation can also be assessed in tumor biopsies obtained under local or general anesthesia (Fig. 1.2). Intravenously administered bio-reductive chemical markers, such as the nitroimidazoles pimonidazole, CCI-103F and EF5, are reduced under hypoxic conditions and subsequently stain hypoxic tumor areas 41,42. Endogenous hypoxia-related markers are physiologically involved in the tumor cell response to hypoxia. These include proteins such as hypoxia-inducible factor 1α (HIF-1α), glucose transporter 1 (Glut-1) and carbonic anhydrase IX (CA-IX) 43-46. Clonogenic repopulation can be assessed using the exogenous S-phase specific thymidine analogues
bromodeoxyuridine and iododeoxyuridine (IdUrd) or by staining endogenous proteins involved in the cell cycle, e.g., Ki-67 and proliferating cell nuclear antigen \(^{47-50}\).

These methods, again, have downsides: tumor biopsies are only obtained from the accessible part of the tumor and may not always be representative of the intra-tumor heterogeneity. Furthermore, immunohistochemical staining is typically performed on tumor sections of 5 \(\mu\)m thickness, cut from the tumor biopsy, and thus again limits the spatial information. Finally, repetitive tumor assessment is hampered and only feasible in accessible squamous cell carcinomas of the oral cavity or oropharynx, due to the invasive nature of biopsy acquisition and the requirement of general anesthesia in most of the patients.

In conclusion, the limitations of methods discussed above call for non-invasive anatomical and/or functional imaging techniques.

### 1.5 Non-invasive imaging modalities for tumor characterization and response assessment

#### 1.5.1 Anatomical imaging

Non-invasive anatomical imaging modalities have the advantage of depicting the entire tumor, potentially at numerous time-points. For tumors of the larynx and hypopharynx, computed tomography (CT) with intravenous iodine contrast agent is the imaging modality of choice. It enables localization and delineation of the primary tumor, (metastatic) lymph nodes, and surrounding tissues that are possibly invaded by the primary tumor, such as the thyroid cartilage. Magnetic resonance imaging (MRI) with intravenous administration of gadolinium is the preferred modality for tumors of the oral cavity and oropharynx. MRI delivers supreme soft tissue contrast facilitating, for example, the differentiation between deep tongue musculature and infiltrative tumor tissue. Along with a thorough clinical examination of the head and neck region, CT and MRI form the cornerstones for primary tumor staging and delineation (www.oncoline.nl). Anatomical imaging modalities such as CT and MRI have been used for tumor response evaluation during and after treatment \(^{51,52}\). However, tumor shrinkage during treatment occurs relatively slow, at a median rate of approximately 2% per day \(^{51}\). Furthermore, CT and MRI are both hampered by anatomical changes occurring during treatment, e.g., edema or development of necrosis, and by post-therapeutic distorted anatomy caused by tumor regression. Functional imaging based on CT, MRI, or positron emission tomography (PET) may not only assist the pre-treatment localization and delineation of the tumor, but may also define biological response profiles.
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1.5.2 Functional imaging

There are different types of functional imaging modalities, some of which have been developed based on anatomical imaging techniques. Dynamic contrast enhanced CT (DCE-CT) provides information about blood flow, blood volume, capillary permeability and micro-vessel density. Tumor perfusion assessed by DCE-CT was shown to be an independent predictor for local failure in a large panel of head and neck tumors. From dynamic contrast enhanced MRI (DCE-MRI) and blood oxygen level dependence (BOLD) MRI, data on tissue perfusion and tumor oxygenation can be extracted. Diffusion-weighted MRI relies on thermal motion of water molecules occurring during cell swelling, tumor lysis and necrosis. Magnetic resonance spectroscopy (MRS) provides chemical information about tissue metabolites. The advantages of these imaging modalities are: broad availability, high resolution (DCE-CT and DCE-MRI), possibility of quantification and repetitive imaging before or during the course of treatment, and incorporation in adaptive radiotherapy techniques. The disadvantages include the indirect nature of the measurements, the lack of metabolic information (CT, MRI) and a poor resolution in case of MRS.

PET is a highly sensitive imaging modality with a relatively good temporal, but inferior spatial resolution. The administered radionuclide emits positrons that annihilate with electrons producing two photons simultaneously traveling in opposite directions. These photons are subsequently detected on a ring of crystals arranged around the volume of interest. Coincidental events result in a “sinogram” that is finally reconstructed for image display. PET enables in vivo imaging of biologically active molecules, such as peptides, hormones, metabolites, and pharmaceuticals. A variety of tumor characteristics, i.e., glucose metabolism, hypoxia, proliferation and receptor expression can be non-invasively visualized with PET using specific radiopharmaceuticals. Furthermore, PET may support the localization and delineation of the primary tumor and possibly of the metastatic cervical lymph nodes. Additionally, the PET signal may hold important information on overall tracer uptake within the lesion and on its intratumoral heterogeneity. The introduction of integrated PET-CT scanners has further broadened the possibilities for accurate tumor localization and characterization.

18F-fluorodeoxyglucose (18FDG) is the most widely used PET tracer in oncology, applied for tumor detection, staging, and treatment response monitoring. In this era of high-precision radiotherapy, 18FDG-PET can provide important complementary information for treatment planning in head and neck tumors, facilitating normal tissue sparing and dose escalation to resistant tumor subvolumes. However, primary squamous cell carcinomas of the head and neck are often ulcerative lesions accompanied by reactive cervical lymph nodes. Glucose consumption of inflammatory cells results in false-positive 18FDG-PET readings and limits the value of this tracer for this tumor entity. Other PET tracers are available that more specifically image features of the tumor that are related to radiation treatment response.
Figure 1.1  Immunohistochemical staining of a head and neck squamous cell carcinoma. A: Tumor cell proliferation (red) and hypoxia (green) relative to blood vessels (yellow). B: Radiation-induced DNA double-strand breaks (red/yellow) as a measure of intrinsic radiosensitivity; hypoxia is shown in green, vasculature in blue.

Figure 1.2  A: Pseudo-colored image of a biopsy obtained from a laryngeal carcinoma immunohistochemically stained for vessels (blue), tumor cell hypoxia (pimonidazole, green) and proliferation (iododeoxyuridine, red). B: The enlarged detail illustrates the typical distribution of cells, with proliferating tumor cells lining the vasculature, tumor cell hypoxia at a certain distance from the vessels (typically 150 μm), and finally necrosis (N).
18F-fluoromisonidazole (18FMISO) is a nitroimidazole widely used for imaging tumor cell hypoxia in squamous cell carcinomas of the head and neck 63,64. It was shown to have both prognostic and predictive value in this tumor entity 65. Furthermore, dose escalation studies directed against 18FMISO positive subvolumes or pixels have demonstrated technical feasibility 64,66,67.

Tumor cell proliferation can be non-invasively imaged using 3'-deoxy-3'-18F-fluorothymidine (18FLT) PET. This radionuclide reflects the activity of thymidine kinase 1 (TK-1), a principal enzyme in the salvage pathway of DNA synthesis 68. Only one study had applied this tracer to head and neck squamous cell carcinomas before we initiated our clinical studies 69. This study investigated the potential of 18FLT-PET for tumor detection, but did not further explore the functional information obtained with 18FLT.

1.6 Outline of the thesis

This thesis deals with the non-invasive characterization of squamous cell carcinomas of the head and neck using PET, with emphasis on tumor cell hypoxia and proliferation. Validation of these PET tracers against histopathology, using xenograft tumor models or human tumor resection specimen, is a pre-requisite before their wide clinical introduction. Next, patient selection for treatment modification based on these PET findings will be the aim of clinical studies. Further investigations will explore the role of specific PET tracers for response monitoring and treatment adaptation.

In chapter 2 an introductory overview regarding the role of PET in radiation therapy planning for head and neck tumors is given. 18FDG-PET can be used for staging purposes, for gross tumor volume delineation, and for dose escalation to tumor subvolumes with high tracer uptake. Additionally, several PET tracers are available that non-invasively image biological tumor characteristics reflecting radiation resistance mechanisms and thus offer potential for tailored radiation therapy.

Tumor cell hypoxia is one of the three major resistance mechanisms in head and neck carcinomas. Non-invasive imaging modalities depicting endogenous hypoxia-related markers, such as carbonic anhydrase IX (CA-IX), are being developed. However, before these tracers can be used for PET imaging, pre-clinical testing using biodistribution and autoradiography is compulsory. In chapter 3, we examined the radioactively labeled monoclonal antibody against CA-IX, 111In-G250, in a panel of squamous cell carcinoma xenograft lines. Biodistribution of this radioactive tracer and CA-IX immunohistochemistry were compared to hypoxia detected by pimonidazole immunohistochemistry. The ultimate aim was to develop and test this tracer for applicability in clinical PET.
\(^{18}\)FMISO is the most widely used PET tracer for the detection of hypoxia in head and neck tumors \(^{63-67,70,71}\). However, in contrast to pimonidazole immunohistochemistry, little was known about the microregional distribution of \(^{18}\)FMISO. **Chapter 4** describes a validation study of \(^{18}\)FMISO autoradiography against pimonidazole immunohistochemistry in ten head and neck squamous cell carcinoma xenograft lines.

A subsequent study was conducted to assess whether changes in oxygenation status were also accurately detected by \(^{18}\)FMISO autoradiography. This is described in **chapter 5**. In three human xenograft lines (head and neck squamous cell carcinoma and glioblastoma), alterations in tracer uptake after increasing and decreasing the hypoxic tumor state were assessed, comparing pimonidazole immunohistochemistry and \(^{18}\)FMISO autoradiography.

Prior to assessing the potential role of \(^{18}\)FLT-PET for monitoring tumor cell proliferation in head and neck cancer, a clinical validation study was performed (**chapter 6**). Seventeen patients with oral cavity tumors underwent \(^{18}\)FLT-PET before surgical tumor resection. The exogenous S-phase marker IdUrd was administered prior to surgery, and tumor samples were immunohistochemically stained for IdUrd and TK-1.

Based on ten patients undergoing selective or (modified) radical neck dissection, we assessed the value of \(^{18}\)FLT-PET for the detection of cervical lymph node metastases. Results of this study are presented in **chapter 7**.

Finally, **chapter 8** shows the findings of a study on ten oropharyngeal cancer patients undergoing three consecutive \(^{18}\)FLT-PET-CT scans, one prior to and two during the course of radiotherapy with or without concomitant chemotherapy.

**Chapter 9** provides a general discussion including future perspectives. A summary of the work is given in **chapter 10**.

### 1.7 References


Innovations in radiotherapy planning of head and neck cancers: role of PET imaging

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Chapter 2

Abstract

Modern radiotherapy techniques heavily rely on high quality medical imaging. Positron emission tomography (PET) provides biological information about the tumor, complementary to anatomical imaging. Integrated PET-CT has found its way into the practice of radiation oncology and $^{18}$F-fluorodeoxyglucose ($^{18}$FDG) PET is being introduced for radiotherapy planning. The functional information possibly augments accurate delineation and treatment of the tumor and its extensions while reducing the dose to surrounding healthy tissues. In addition to $^{18}$FDG, other PET tracers are available for imaging specific biological tumor characteristics determining radiation resistance. For head and neck cancer, the potential gains of PET imaging are increasingly being recognized. This review describes the current role and future perspectives of PET for selection and delineation of radiotherapy target volumes and for biological tumor characterization in this tumor entity. Furthermore, the potential role of PET for early response monitoring, treatment modification and patient selection is addressed in this review.
2.1 Innovations in radiotherapy for head and neck tumors

The field of radiation oncology changed dramatically with the wide introduction of computer optimized intensity-modulated radiation therapy (IMRT) in the 1990's. IMRT is based on the use of numerous radiation beams with optimized non-uniform intensities resulting from inverse treatment planning. The algorithm for beam fluence calculations is guided by dose-volume objectives for the target volume and organs at risk delineated by the radiation oncologist. IMRT can thus achieve much better dose conformity than conventional radiotherapy techniques. With this technique, different dose prescriptions to multiple target sites can be delivered. It also facilitates boosting of the primary tumor to high radiation doses while reducing the dose to radiation sensitive tissues adjacent to the tumor. Due to the highly conformal dose distribution and steep dose gradients used in IMRT, knowledge about the localization and boundaries of the primary tumor and of the cervical lymph node metastases is crucial. For this purpose, biological imaging using positron emission tomography (PET) may augment traditional imaging methods such as computed tomography (CT) and magnetic resonance imaging (MRI) (Fig. 2.1).

2.2 PET for highly accurate radiation treatment planning

2.2.1 Identification of radiotherapy targets based on $^{18}$FDG-PET

In a recent issue of this journal, Fletcher et al. reviewed the available literature and a multidisciplinary expert panel developed recommendations on the use of $^{18}$FDG-PET in oncology practice. These recommendations on the use of $^{18}$FDG-PET for the detection and staging of head and neck tumors are briefly summarized here.

The expert panel concluded that $^{18}$FDG-PET should not be added to conventional anatomical imaging in the routine diagnostic work-up of primary head and neck tumors. This conclusion was drawn as the available data were too uncertain as to whether $^{18}$FDG-PET can determine the anatomic extent of the primary tumor more accurately than CT or MRI.

Regarding the detection of cervical lymph node metastases, Fletcher et al. concluded that $^{18}$FDG-PET has a higher sensitivity, specificity, positive predictive value and negative predictive value compared to CT and MRI. Therefore, its use in routine local staging was recommended. However, this recommendation did not incorporate the findings of the meta-analysis by Kyzas et al. that was published shortly thereafter. This meta-analysis reviewed 35 studies using $^{18}$FDG-PET for the pretreatment evaluation of the lymph node status. The authors concluded that there was no solid evidence to support the routine application of $^{18}$FDG-PET, as the sensitivity and specificity only improved by 5-7% compared to conventional imaging modalities. In the subset of studies only enrolling patients without clinically apparent cervical lymph node metastases, $^{18}$FDG-PET may have a slightly better diagnostic accuracy.
metastases, the sensitivity was only 50% and not better than conventional imaging methods, specifically ultrasound with fine needle cytology. From these contradictory recommendations it is clear that this is an unresolved issue that requires further study.

For the detection of distant metastases, the net benefit of using $^{18}$FDG-PET was reported to be still uncertain. Functional imaging might be beneficial in patients with advanced-stage disease, in whom the odds of having distant metastases are greater. In these patients, the $^{18}$FDG-PET findings may alter the treatment intention from curative to palliative and thus impact on the total dose and fractionation scheme. Additionally, it may reduce treatment-related side-effects in those patients, as the selected treatment volume is often confined to the primary tumor and/or metastatic lymph nodes causing discomfort or pain. The role of $^{18}$FDG-PET(-CT) for the early detection of recurrent disease is addressed elsewhere.

It can be concluded that there is only a modest role for $^{18}$FDG-PET in the routine diagnostic workup and staging of head and neck cancer patients. However, this does not disqualify $^{18}$FDG-PET as a potentially useful and complementary tool for accurate radiotherapy target volume delineation and customized dose delivery.

**Figure 2.1** $^{18}$FLT-PET-CT scan for image-guided high-precision radiation treatment planning in an oropharynx carcinoma
Figure 2.2  (legend on page 30)

Figure 2.3  (legend on page 30)
In this era of high-precision radiotherapy, accurate tumor volume delineation with respect to tumor boundaries, shape and volume is crucial. Target volume delineation is primarily based on anatomical information of the tumor and affected lymph nodes. A thorough physical examination of the head and neck forms the basis for assessment of tumor extensions, especially for superficially spreading mucosal tumors. Anatomical imaging using CT and/or MRI provides important complimentary information by depicting distorted anatomy and regions of abnormal contrast enhancement. For oral cavity and oropharyngeal carcinomas, MRI is the preferred imaging modality as it achieves a better soft-tissue contrast.

There are a number of potential advantages of using $^{18}$FDG-PET for target volume delineation. $^{18}$FDG-PET may reduce the interobserver variability in gross tumor volume (GTV) delineation, reduce the size of the GTV, identify tumor areas or lymph nodes missed by CT or MRI, and it may identify parts of the GTV potentially requiring an additional radiation dose. However, the use of $^{18}$FDG-PET also bears some disadvantages: the limited spatial resolution, the lack of a standardized method for signal segmentation, and false-positive $^{18}$FDG-PET readings caused by inflammation.

A reduction of the interobserver variability has been demonstrated for non-small cell lung cancer when $^{18}$FDG-PET was incorporated in GTV delineation. $^{18}$FDG-PET may reduce the interobserver variability in gross tumor volume (GTV) delineation, reduce the size of the GTV, identify tumor areas or lymph nodes missed by CT or MRI, and it may identify parts of the GTV potentially requiring an additional radiation dose. However, the use of $^{18}$FDG-PET also bears some disadvantages: the limited spatial resolution, the lack of a standardized method for signal segmentation, and false-positive $^{18}$FDG-PET readings caused by inflammation.
radiation oncologists and two neuroradiologists delineated 16 head and neck cancer patients. On average, the GTVs based on $^{18}$FDG-PET-CT were larger than the corresponding CT-based volumes. Furthermore, the authors observed a large discrepancy between the GTV delineation of the two radiation oncologists, one delineating larger volumes on CT, the other one on $^{18}$FDG-PET-CT. One important difference between these two studies relates to thresholding of the $^{18}$FDG-PET signal: Ciernik et al. chose a fixed threshold-level of 50% of the maximum signal intensity, whereas Riegel et al. used a discretionary window-level setting. The issue of segmentation of the $^{18}$FDG-PET signal from background will be addressed in one of the next paragraphs.

A reduction of the GTV using $^{18}$FDG-PET has been demonstrated in a landmark study including laryngeal cancer patients. The authors investigated the role of co-registered CT, MRI and $^{18}$FDG-PET in GTV delineation of patients undergoing laryngectomy. Compared to the reference surgical specimen, $^{18}$FDG-PET was closest to depict the true tumor volume. All modalities overestimated the extension of the tumor, $^{18}$FDG-PET with an average of 29%, CT with 65%, and MRI with 89%. However, all three imaging modalities, including $^{18}$FDG-PET, failed to identify a small fraction of the macroscopic tumor (approximately 10%), mainly consisting of superficial mucosal extensions.

Before PET-based GTVs can reliably and reproducibly be incorporated into high-precision radiotherapy planning, operator-independent segmentation tools have to be developed and validated. Simple visual interpretation of the PET signal is most commonly applied but highly operator dependent, as it is very susceptible to the window-level settings of the images and interpretation differences. This is why research groups have explored more objective methods, such as isocontouring based on a fixed standardized uptake value (SUV), e.g., of 2.5, or thresholds acquired through phantom experiments such as a fixed threshold of the maximum tumor signal intensity (40% or 50%) or a fixed threshold of the maximum tumor signal intensity (40% or 50%). Daisne et al. used a variable threshold adaptive to the signal-to-background ratio (SBR method) in their study on laryngeal cancer patients. Recently, the same group published a new gradient-based segmentation tool based on watershed transform and hierarchical cluster analysis and validated this in an adaptive biological image-guided planning study. Shortly thereafter, van Dalen et al. published an iterative background-subtracted relative-threshold level (RTL) method validated in patients with liver metastases. The optimal RTL thereby depends on the lesion size, but not on the signal-to-background ratio. A recent study in 78 head-and-neck cancer patients compared five commonly used methods of $^{18}$FDG-PET signal segmentation (visual interpretation, 40% and 50% of the maximum tumor signal intensity, fixed SUV of 2.5 and the SBR method; Fig. 2.2 and Table 2.1). The results showed that the volume and the shape of the resulting GTV were heavily influenced by the choice of the segmentation tool.
Visual interpretation of the PET signal yielded volumes close to those of CT-based GTV delineation, whereas all automated segmentation methods resulted in significantly smaller GTVs than the GTVs based on clinical information and CT alone. Furthermore, in a large percentage of patients (between 29% and 64%, depending on the segmentation tool used) more than 20% of the $^{18}$FDG-PET-based GTV was located outside the GTV based on clinical information and CT. This suggests that tumor could be identified by $^{18}$FDG-PET that was missed using the standard methods of GTV delineation. However, in the absence of histological validation it is unknown in what percentage of cases this was caused by peritumoral inflammation, resulting in a false-positive reading of the $^{18}$FDG-PET signal.

Most of the discussed data are based on theoretical delineation studies using operator-dependent or -independent segmentation tools. Only the study by Daisne et al. validated their results against histopathology. It is obvious, that additional validation studies are needed as well as carefully designed clinical trials to address the issue of safety (side-effects) and the clinical impact (locoregional control, survival) of incorporating PET for GTV delineation. However, it will be at least challenging to design and conduct such trials while PET-CT is increasingly incorporated into clinical practice on the basis of nonrandomized clinical studies in often relatively small patient populations.

Thus far, most delineation studies in head and neck cancer incorporating $^{18}$FDG-PET have concentrated on the primary tumor. This is probably due to the fact that CT-based delineation of metastatic lymph nodes is usually less prone to error due to better discrimination from the surrounding fatty tissue. However, this can be more difficult in cases with large, matted nodes. As discussed in the previous section, $^{18}$FDG-PET might be helpful in these situations, although one should be aware of the possibility of negative $^{18}$FDG-PET readings in necrotic parts of the lymph node.

Apart from more accurate target volume delineation for radiotherapy planning purposes, PET may identify parts of the GTV potentially requiring additional radiation doses. Assuming that $^{18}$FDG uptake represents tumor cell density, $^{18}$FDG-PET can be used to direct dose escalation to $^{18}$FDG-avid subvolumes of the tumor. The feasibility of
this approach was demonstrated in various theoretical planning studies applying either uniform dose distribution or voxel-intensity-based IMRT \(^{21,22}\). The former method delivers a uniform escalated dose to an \(^{18}\)FDG-avid subvolume within the CT-based target volume. This approach was pioneered by Schwartz et al. escalating total dose up to 75 Gy in a theoretical planning study involving 20 head and neck cancer patients \(^{21}\). With voxel-intensity based IMRT, the \(^{18}\)FDG signal-intensity in the PET-voxel is proportionally related to the dose prescribed to that voxel, \textit{i.e.}, the higher the PET-signal, the higher the prescribed dose \(^{22}\). Both methods are alternatives for boosting \(^{18}\)FDG-PET subvolumes inside a CT-based planning target volume. The clinical feasibility of dose escalation using a uniform dose distribution was recently proven in a phase I clinical trial \(^{23}\). Forty-one head and neck cancer patients were treated with IMRT to two dose levels of 72.5 Gy and 77.5 Gy using a simultaneous integrated boost (SIB). With this technique, the escalated dose is delivered simultaneously with the lower dose to the low risk areas as opposed to sequentially where the boost is delivered at the end of the treatment. Acute toxicity (dysphagia \textgreater; grade 3) occurred in 50\% of patients at both dose levels and dose-limiting toxicity was observed in two patients at the lower dose level and one patient at the higher. The authors concluded that PET-guided dose escalation appeared to be well-tolerated with high local control rates in both the lower and higher dose groups of 85\% and 87\%, respectively, at 1 year of follow-up (Fig. 2.3)\(^{23}\). During the course of radiotherapy, the tumor volume gradually decreases and one might consider adjusting the GTV and ultimately the radiotherapy dose distribution accordingly. This could facilitate sparing of normal tissues. For example, during the treatment course for oropharyngeal tumors, the parotid gland is shifted centrally towards the high-dose region by tumor shrinkage and weight loss of the patient \(^{24}\). As a result, a larger part of the parotid gland is potentially irradiated to a higher dose, which may result in a higher incidence and greater severity of xerostomia. “Adaptive image-guided radiotherapy” using repetitive PET-CT scanning during the course of treatment is a promising approach to adjust treatment volume and dose distribution. This has been recently demonstrated in a proof of principle study \(^{18}\). Throughout the course of radiotherapy, the GTVs based on \(^{18}\)FDG-PET significantly decreased and were at all times smaller than those defined using pre-treatment CT and MRI. Radiation treatment planning based on \(^{18}\)FDG-PET and volume adaptation progressively reduced the irradiated volumes by 27\%-42\% (\(V_{90}-V_{100}\)) compared to traditional CT-based treatment plans obtained prior to treatment. Disappointingly, this volume reduction only marginally impacted on the doses to the organs at risk, such as the parotid gland. Adaptive \(^{18}\)FDG-PET-guided radiotherapy nevertheless may be an attractive approach, especially for dose escalation strategies.

In conclusion, \(^{18}\)FDG-PET can provide important complementary information for radiotherapy planning in head and neck cancer. Potentially, the GTV can be reduced based on the PET information, which facilitates sparing of nearby normal tissues and
allows dose escalation to relatively small subvolumes. Furthermore, biological imaging using $^{18}$FDG-PET may identify areas of tumor spread not recognized by CT or MRI, which can potentially improve the accuracy of GTV definition. However, to address the clinical value and possible shortcomings of these concepts, additional histological validation studies and properly designed clinical studies are needed.

Table 2.1 Common terminology used in radiation therapy

<table>
<thead>
<tr>
<th>Terminology</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gross Tumor Volume (GTV)</td>
<td>Macroscopic tumor volume as detected by clinical examination and anatomical imaging (ultrasound, CT, MRI, PET)</td>
</tr>
<tr>
<td>Clinical Target Volume (CTV)</td>
<td>Gross Tumor Volume with margin added for sub-clinical microscopic spread</td>
</tr>
<tr>
<td>Planning Target Volume (PTV)</td>
<td>Clinical Target Volume with margin added for organ motion and setup inaccuracy</td>
</tr>
<tr>
<td>Treatment Volume</td>
<td>Tissue volume treated to substantial radiation dose (typically larger than PTV)</td>
</tr>
<tr>
<td>Metabolic Tumor Volume</td>
<td>Metabolically active tumor volume as detected by biological imaging, e.g., $^{18}$FDG-PET</td>
</tr>
<tr>
<td>Hypoxic Tumor Volume</td>
<td>Hypoxic tumor volume as detected by biological imaging, i.e., hypoxia or hypoxia-related PET tracers ($^{18}$FMISO, $^{18}$FAZA or $^{18}$FETNIM) or BOLD-MRI</td>
</tr>
</tbody>
</table>

Recently, the first results on clinical treatment outcome after integration of $^{18}$FDG-PET-CT data into IMRT planning have been published. In a case-control-study, Rothschild et al. compared 45 patients with stage IV-A pharyngeal carcinomas treated with $^{18}$FDG-PET-CT-based IMRT with a matched historical cohort receiving standard three-dimensional conformal radiotherapy. The 2-year overall survival and event-free survival rates of patients treated with $^{18}$FDG-PET-CT-based IMRT were 91% and 80%, and significantly better than for the control group. In a similar study, Vernon et al. reported 2-year overall survival and disease-free survival rates of 83% and 71% for 42 patients with head and neck cancer of various stage and subsites. Toxicity profiles in this second study were reported as favorable.

Even though these initial results are encouraging, they must be interpreted cautiously because they are based on small and heterogeneous patient populations, they only have short follow-up periods, and they use historical controls. Furthermore, it remains unclear from both studies whether the suggested improvements in tumor control must be attributed to improved radiotherapy techniques, or the introduction of $^{18}$FDG-PET-CT or to other factors.
2.3 Imaging biological tumor characteristics relevant for radiation treatment response

Three major tumor characteristics adversely affect treatment outcome and prognosis after radiation therapy: tumor cell hypoxia, repopulation during the course of treatment and intrinsic radioresistance. These factors largely determine the outcome of radiotherapy in terms of local and regional tumor control, but ultimately also the risk of distant metastases and survival. PET enables non-invasive biological profiling of the tumor prior to and during radiation treatment with the potential to tailor therapy according to individual characteristics.

2.3.1 Hypoxia

Hypoxia is a feature of many solid tumors and in particular squamous cell carcinomas of the cervix and the head and neck. Tumor cell hypoxia can result from two mechanisms: limited diffusion capacity of oxygen due to a large distance from the supplying blood-vessel (chronic hypoxia) or impaired perfusion of the supplying vessel due to temporary vasoconstriction or endovascular obstruction (acute hypoxia). Treatment modifications are available, but at the cost of increased morbidity. In order to individualize treatment and to select patients for these treatment modifications, assessment of the tumor oxygenation status is compulsory. In accessible tumors of the head and neck or uterine cervix, this can be done by invasive polarographic electrode measurements or by immunohistochemical staining of markers in tumor biopsies. The advantage of the polarographic electrodes is that the entire tumor can be mapped using multiple tracks. However, its clinical use is limited due to the invasive nature of the procedure, the restriction to accessible tumors and the inability to distinguish between normal, necrotic or tumor tissue. Immunohistochemical staining of tumor biopsies results in high-resolution images that can be analyzed for several endogenous and exogenous markers of interest. Unfortunately, the tumor biopsies are often small and only represent a fraction of the entire tumor. Furthermore, exogenous markers require intravenous administration before biopsy taking. Finally, the acquisition of tumor biopsies often requires the use of general anesthesia, and this procedure is not attractive for repetitive measurement. Non-invasive imaging using PET can provide a spatial map of the intra-tumoral distribution of hypoxia before and during treatment. This information can potentially be used not only as a selection instrument for treatment modification, but also for optimization of radiotherapy planning and delivery.

18F-fluoromisonidazole (18FMISO) is a nitroimidazole PET-tracer that is reduced and bound to cell constituents under hypoxic conditions. In the early 1990's, 18FMISO-PET was applied in several small clinical trials on different primary tumors. Since then, 18FMISO-PET has been extensively used for the detection of hypoxia in head and neck
Role of PET in radiotherapy

tumors\textsuperscript{38-44}. Importantly, in head and neck cancer it was shown that the level of hypoxia depicted by \textsuperscript{18}FMISO-PET prior to treatment was correlated with locoregional failure\textsuperscript{38,42,43}. Apart from its prognostic value, Rischin et al. published data supporting the predictive value of \textsuperscript{18}FMISO-PET\textsuperscript{42}. They performed \textsuperscript{18}FMISO-PET scans in patients with advanced stage head and neck carcinomas that were treated with radiotherapy and concurrent chemotherapy alone or combined with a hypoxic cytotoxin. Patients with hypoxic primary tumors treated with the additional cytotoxin experienced significantly less local failures compared to patients treated with chemotherapy alone (zero of eight patients versus six of nine). Furthermore, the absence of hypoxia on \textsuperscript{18}FMISO-PET was associated with a low risk of locoregional failure when treated with chemotherapy alone\textsuperscript{42}. \textsuperscript{18}FMISO-PET can thus serve as a predictive tool allowing treatment selection based on biological tumor characteristics. Ultimately, reduction of side effects in patients not benefiting from treatment modification will be feasible.

Apart from tumor characterization, first attempts were made to delineate a biological target volume and to escalate the dose to the primary tumor based on \textsuperscript{18}FMISO-PET\textsuperscript{45-47}. Two theoretical planning studies proved the feasibility of dose escalation to the \textsuperscript{18}FMISO-PET detected hypoxic subvolume using IMRT\textsuperscript{45,46}. Rajendran et al. demonstrated that using an IMRT technique, the dose to the \textsuperscript{18}FMISO-PET detected hypoxic subvolume could be escalated by an additional 10 Gy\textsuperscript{46}. Lee et al. achieved a dose of 84 Gy in hypoxic areas without exceeding the normal tissue tolerance\textsuperscript{45}. Their attempt to further escalate the dose to 105 Gy in hypoxic regions was only successful in one of the two plans studied. In a third study, Thorwarth et al. compared IMRT planning with dose painting by numbers based on dynamic \textsuperscript{18}FMISO-PET data\textsuperscript{47}. Thereby, spatially variant doses are delivered to the tumor according to dose-escalation factors determined on the bases of the dynamic \textsuperscript{18}FMISO-PET scan. Applying this approach, the tumor control probability was increased from 56\% to 70\%, while maintaining the same level of toxicity\textsuperscript{47}. However, one has to be cautious interpreting the data as the number of patients included in this study was small.

Until now, clinical experience with hypoxic PET tracers other than \textsuperscript{18}FMISO is limited. \textsuperscript{60}Cu(II)-diacetyl-bis(N\textsuperscript{4}-methylthiosemicarbazone) (\textsuperscript{60}Cu-ATSM) was introduced into the clinic after successful pre-clinical studies demonstrating a strong correlation between tracer uptake and low pO\textsubscript{2} levels\textsuperscript{48}. It was the first hypoxia-related PET tracer for which the potential use of a selective boost to the hypoxic subvolume was illustrated\textsuperscript{49}. However, partly due to its limited specificity, especially if imaging is performed at early time points after administration, this compound did not find its way in larger scale clinical studies.

\textsuperscript{18}F-fluoroerythronitroimidazole (\textsuperscript{18}FETNIM), \textsuperscript{18}F-fluoroazomycin arabinoside (\textsuperscript{18}FAZA) and \textsuperscript{18}F-2-(2-nitroimidazol-1-yl)-N-(3,3,3-trifluoropropyl)-acetamide (\textsuperscript{18}F-EF3) are
members of a new generation nitroimidazoles. $^{18}$FETNIM showed a higher and more heterogeneously distributed tracer uptake in tumors than in adjacent neck muscle. Furthermore, a high uptake of $^{18}$FETNIM prior to radiation therapy was associated with a trend towards poor overall survival. $^{18}$FAZA has similar tracer characteristics compared to $^{18}$FETNIM, and was proven feasible and of sufficient quality for clinical use in head and neck cancer patients. Grosu et al. incorporated $^{18}$FAZA-PET in radiation treatment planning and detected hypoxic subvolumes of different size and distribution (representing on average 11% of the primary tumor volume and 8% of the metastatic lymph node volume). Dose escalation to 80.5 Gy in $^{18}$FAZA-PET detected hypoxic areas was shown feasible. $^{18}$F-EF3 was used in a phase I study in head and neck cancer patients. In this study, the use of this tracer was shown to be safe, but the number of advanced stage tumors showing increased tracer uptake was disappointingly low.

In summary, although numerous hypoxic or hypoxia-related PET-tracers are available for clinical use, their prognostic and predictive value needs to be assessed in larger clinical studies before implementation for patient selection. Preferably, the PET-tracer used must also visualize changes in the oxygenation status caused by treatment modifications counteracting hypoxia, such as carbogen breathing. More importantly, the concept of dose painting to hypoxic subvolumes either by uniform doses or by dose-painting by numbers is still subject of intense debate. There are major concerns about the spatial resolution of hypoxic PET imaging when compared to the distribution and fluctuation of tumor cell hypoxia at the microregional level. In this context, we investigated ten different head and neck carcinoma xenograft tumor lines using $^{18}$FMISO autoradiography and pimonidazole immunohistochemistry (Fig. 2.4). We found that the pattern of the $^{18}$FMISO signal depended on the distribution of hypoxia at the microregional level. In five xenograft tumor lines, a significant correlation between the mean $^{18}$FMISO and pimonidazole signal intensity was found and this depended on the underlying microarchitecture. This indicates that one should be cautious when studying small tumor subvolumes for dose escalation.

Apart from different distribution patterns of hypoxia at the microregional level, one has to consider that the oxygenation status changes during the course of radiotherapy, which makes repetitive PET-imaging before and during treatment compulsory. Finally, the question regarding the radiation dose levels required for effective elimination of the radioresistant subpopulations remains unsolved.

### 2.3.2 Tumor cell proliferation

The major limitations of $^{18}$FDG-PET in oncology are false-positive readings due to tracer uptake in inflammatory tissue or reactive lymph nodes. Therefore, PET tracers that more specifically image DNA-synthesis are currently being developed and tested.
Tumor cell proliferation during the course of therapy adversely affects radiation treatment outcome and prognosis in squamous cell carcinomas of the head and neck. 3'-deoxy-3'-18F-fluorothymidine (18FLT) is a tracer that reflects the activity of thymidine kinase 1, a principal enzyme in the salvage pathway of DNA synthesis. The 18FLT-PET signal is more specific for actively dividing tumor cells compared to 18FDG. Inflammatory cells in the proximity of the tumor consume glucose and thus cause false-positive 18FDG-PET readings. However, as these immune response cells are terminally differentiated, the DNA synthesis rate and therefore the 18FLT uptake are not increased. 18FLT-PET was validated against histopathology in a variety of solid tumors including breast, lung and sarcoma. In soft-tissue sarcoma, Cobben et al. found a significant correlation between the SUV values and labeling index of the proliferation marker Ki-67. In addition, 18FLT-PET was able to distinguish low-grade from high-grade soft-tissue sarcomas. In breast tumors, Kenny et al. reported a strong correlation between SUV values and Ki parameter of dynamic 18FLT-PET and staining of Ki-67. Finally, Yap et al. also observed a significant correlation between 18FLT uptake in non-small cell lung cancer lesions and the Ki-67 labeling index.

In primary head and neck tumors, this promising compound has thus far only been applied to primary laryngeal tumors. Currently, validation of 18FLT-PET in a large series of squamous cell carcinomas of the head and neck is ongoing at our center. For the detection of cervical lymph node metastases, first results demonstrated that 18FLT-PET is not suitable in this tumor entity. A high rate of false positive findings caused by 18FLT uptake in the germinal centers of reactive lymph nodes resulted in a low specificity and a low positive-predictive value (17% and 38%, respectively).

Until present, adaptive image-guided radiotherapy has been based on repetitive PET-scanning using 18FDG. As the treatment course progresses, the obtained 18FDG-PET-signal is heavily influenced by the inflammatory response of tumor-surrounding tissues leading to an increased background activity. As a result, segmentation of the PET signal for tumor delineation purposes becomes increasingly difficult. The use of a proliferation-specific PET tracer, such as 18FLT, may be a solution to this problem. During the course of therapy, the reduction in the proliferative activity of the primary tumor can be accurately imaged by 18FLT, not disturbed by increased tracer uptake in surrounding inflammatory tissue. At present, our group is assessing the changes in the 18FLT-PET-signal during therapy in patients with squamous cell carcinomas of the head and neck treated with radiotherapy alone or with concomitant chemotherapy (Fig. 2.5). The predictive potential of this approach and applicability for tailored treatment are subject of investigation.
2.3.3 Perfusion, protein synthesis and others

Another significant tumor characteristic strongly related to tumor cell hypoxia is tumor blood perfusion. Hypoxia is a strong stimulus for neovascularization, but many newly formed vessels are of poor quality with severe structural and functional abnormalities. Despite increased vascular density, the impaired functionality of blood vessels may result in deprivation of oxygen and nutrients.

Therefore, an imaging tool for assessment of tumor blood flow may provide important information relevant for radiotherapy responsiveness. Lehtio et al. used $^{15}$O-labeled water ($^{15}$O-H$_2$O) and $^{18}$FETNIM for imaging of perfusion and hypoxia in 21 patients with head and neck cancer. Preliminary results from this small study indicated an association between tumor perfusion and radiation treatment outcome.

$^{18}$F-fluoroethyl-L-tyrosine ($^{18}$FET) and l-methyl-$^{11}$C-methionine ($^{11}$C-MET) are amino acid analogues used to visualize cellular amino acid uptake or protein synthesis. $^{18}$FET may be useful in differentiating tumor from post-treatment inflammatory tissue as it is not taken up by inflammatory cells. Several studies compared $^{18}$FET- with $^{18}$FDG-PET in squamous cell carcinomas of the head and neck and confirmed the specific uptake of $^{18}$FET by malignant cells with histopathology. The specificity of $^{18}$FET-PET was found to be superior to $^{18}$FDG-PET (95-100% versus 63-79%), but the sensitivity of the amino acid tracer was significantly lower (64-75% versus 93-95%, respectively). As the SUV values for $^{18}$FET-PET were significantly lower compared to $^{18}$FDG-PET, the new tracer will probably not replace $^{18}$FDG-PET as diagnostic tool but it can provide complementary information for discrimination between tumor and inflammatory tissue. $^{11}$C-MET has a similar sensitivity and specificity compared to $^{18}$FDG-PET. In a delineation study, $^{11}$C-MET was compared to $^{18}$FDG-PET and CT. While $^{18}$FDG-PET yielded significantly smaller GTVs compared to CT, GTVs based on $^{11}$C-MET-PET were not different from CT, probably because of uptake by surrounding normal mucosa and salivary gland tissue. The authors concluded that $^{11}$C-MET has no additional value for target volume delineation in head and neck tumors.

$^{1}$-$^{11}$C-acetate ($^{11}$C-ACE) is suggested to preferentially metabolize to the membrane lipids in tumor cells. In a head and neck cancer staging and radiotherapy planning study, $^{11}$C-ACE-PET detected all primary tumors and 95% of the metastatic lymph nodes, more than $^{18}$FDG-PET and CT/MRI. However, the GTVs derived by $^{11}$C-ACE-PET were 51% larger than those based on $^{18}$FDG-PET. Before $^{11}$C-ACE-PET can be introduced in the radiotherapy planning process, further studies are needed to explain this discrepancy and to clarify the mechanism of tumor uptake. Finally, non-invasive methods to assess the uptake and biodistribution of biological modifiers will be of great value to direct new targeted therapies. Radiolabelled antibodies and small molecules for PET imaging are currently being developed and tested in pre-clinical and early clinical studies.
In conclusion, PET tracers imaging specific biological tumor characteristics offer potential for tailor-made radiation therapy. However, they remain in the research arena until proper clinical validation.

2.4 Technical innovations

Several challenges regarding PET-scanning remain, of which some may be resolved or improved while others cannot. For example, resolution is limited by the distance a positron travels before it annihilates, which is a given fact for a certain radionuclide positron emitter and therefore unchangeable. Furthermore, various developments regarding an increase in the spatial and temporal resolution are ongoing. Currently, the spatial resolution for human PET-scanners is in the order of 5-7 mm compared to 1-3 mm for small animal scanners. New developments regarding the size of the detector crystal, the coincidence timing window and the signal processing have achieved a resolution of 2 mm for the human application (product information Siemens). Through this development, the distortion of the image and its blurring is reduced which may result in an increased precision of tumor delineation.

Integrated PET-MRI scanners combine anatomical with functional imaging and may have a specific impact on head and neck cancer staging and treatment. The potential benefits of integrated PET-CT scanning for the planning procedure of radiotherapy have been discussed in this review. However, for particular subsites of the head and neck region, e.g., oropharyngeal and oral cavity tumors, MRI is the diagnostic imaging modality of choice. In these tumor sites, integrated PET-MRI scanners may further improve the accuracy of gross tumor volume delineation. In addition, dynamic MR studies such as dynamic contrast enhancement MRI and BOLD MRI as well as MR spectroscopy may add complementary functional information.

2.5 General conclusions

$^{18}$FDG-PET is the golden standard for non-invasive functional imaging in oncology. In head and neck tumors, $^{18}$FDG-PET is not recommended for the detection of the primary tumor and its value for metastatic lymph nodes is still a matter of debate. With regard to staging of the primary tumor, $^{18}$FDG-PET may influence the treatment decision if distant metastases or second primary tumors are detected.

For radiotherapy planning in head and neck cancer, $^{18}$FDG-PET can provide important complementary information to CT. Based on the PET information, the volume irradiated to high dose levels may be reduced which facilitates normal structures sparing and dose escalation. However, additional histological validation studies and properly designed clinical studies are needed to address the clinical value and possible shortcomings of this concept. Several PET tracers to image biological tumor characteristics reflecting
radiation resistance mechanisms are available and offer potential for tailored radiation therapy. However, they should be restricted to research purposes until proper clinical validation. In this context, the use of more than one tracer may open new horizons in the future. Finally, technical developments in PET-scanning in general and in the field of head and neck cancer in particular may increase the precision of radiotherapy planning and thus improve tumor control and reduce treatment-related morbidity.

2.6 References

Role of PET in radiotherapy


Comparison of different methods of CA-IX quantification in relation to hypoxia in three human head and neck tumor lines

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Abstract

Purpose. In head and neck cancer, it has been shown that hypoxic tumors respond poorly to therapy. Methods to identify hypoxic tumors are therefore of importance to select patients for oxygenation modifying or other intensified treatments. The aim of this study was to compare tumor cell hypoxia assessed by the hypoxic cell marker pimonidazole (PIMO) with expression of the endogenous hypoxia-related marker carbonic anhydrase IX (CA-IX) in three human head and neck tumor lines.

Material and Methods. Forty-five tumors of three human head and neck tumor lines, SCCNij3, SCCNij59 and MEC82, xenografted in athymic mice, were used. CA-IX was quantified by biodistribution (% injected dose / g tumor) after injecting 3-5 μl 111In-labeled G250 mouse antibody 3 days prior to euthanizing. In a tissue section from the same tumor, fractions of tumor area positive for PIMO, CA-IX and Hoechst 33342 (perfusion marker) were assessed after immunohistochemical staining, using a digital image analysis system.

Results. SCCNij3 and MEC82 were relatively hypoxic tumor lines with fractions of tumor area positive for pimonidazole of 0.16 and 0.15, respectively. SCCNij59 was a better oxygenated tumor line with a PIMO-fraction of 0.03. The three tumor lines showed different levels and patterns of CA-IX immunohistochemical staining, but only in MEC82 there was a good correlation between PIMO-fraction and CA-IX-fraction (r² = 0.92, p<0.0001). Correlations between 111In-G250 uptake and CA-IX-fraction or PIMO-fraction within tumor lines were weak or absent.

Conclusion. Assessment of CA-IX expression depends largely on the techniques and tumor lines used. Furthermore, the immunohistochemical staining pattern of CA-IX relative to PIMO differs between human tumor lines of similar anatomical origin. Therefore, the use of CA-IX as endogenous marker of tumor hypoxia remains questionable.
3.1 Introduction

Hypoxia is one of the major factors contributing to radiotherapy resistance of solid tumors and it leads to adverse treatment outcome. It was shown that assessment of tumor cell hypoxia by means of pimonidazole binding can predict for treatment outcome in head and neck cancer. It is therefore important to develop reliable, preferably non-invasive methods to identify hypoxic tumors enabling selection of patients for oxygenation modifying or other intensified treatments.

Hypoxia can be assessed at the microregional level after administration of bioreductive markers such as 2-nitroimidazole pimonidazole hydrochloride (PIMO) and EF5. However, these markers require intravenous administration. Endogenous hypoxia markers are therefore currently proposed as a promising and convenient way of detecting hypoxia. CA-IX, a member of the carbonic anhydrase family is the most promising endogenous hypoxia-related cell marker of the 15 carbonic anhydrase subtypes. This transmembranic enzyme catalyses the reversible hydration of carbon dioxide to carbonic acid and is involved in acid-base balance and cell-to-cell adhesion. CA-IX is up-regulated under increased cell density and under hypoxic conditions with pO$_2$ levels below 20 mmHg. Therefore, regions with high CA-IX expression are typically peri-necrotic. Interestingly, several studies on solid tumors, including the uterine cervix and head and neck cancer, report overexpression of CA-IX in tumor tissue with absence of CA-IX in corresponding normal tissue. Studies on co-localization between PIMO and CA-IX, however, have revealed conflicting results to date.

Over the past few years, various tools to assess CA-IX expression have been developed, e.g., immunohistochemical visualization in tissue sections and measurement of uptake of radioactive labeled anti-CA-IX antibody in tumor tissue following intravenous administration. G250 is one of the two currently available monoclonal antibodies against CA-IX used in nuclear medicine, and it only binds to the native form of the CA-IX antigen. G250 is gaining interest as antibody for non-invasive molecular imaging methods such as immunohistochemistry, uptake measurement and possibly SPECT and PET analysis of CA-IX.

In the present study, the exogenous hypoxic cell marker PIMO was compared with the endogenous hypoxia-related marker CA-IX. CA-IX was determined immunohistochemically and by assessing the concentration of $^{111}$In-labeled G250 antibody following intravenous administration.

3.2 Material and Methods

Mouse Tumor Model. Twenty-six SCCNij3 tumors, seven SCCNij59 and twelve MEC82 tumors, xenografted in athymic BALB/c nu/nu mice, were used in these experiments. Viable 1 mm$^3$ tumor pieces were implanted subcutaneously. Tumors with a mean
diameter of 6–8 mm were used in the experiments. Animals were kept in a specific-pathogen-free unit in accordance with institutional guidelines. After euthanizing the animals, tumors were snap frozen in liquid nitrogen. All experiments were approved by the Animal Experiments Committee of the Radboud University Nijmegen Medical Centre.

**Monoclonal Antibody cG250.** The isolation and immunohistochemical reactivity of mAb G250 have been described elsewhere. In order to reduce the immunogenicity of the G250 antibody, the chimeric version, mAb cG250, has been developed, which is reactive with the antigen G250, the tumor-associated carbonic anhydrase isoenzyme IX (MN/CA-IX).

**Conjugation, Radiolabeling, and Quality Control.** The conjugation of SCN-Bz-DTPA to mAb cG250 was performed as described by Ruegg et al. with minor modifications. The number of DTPA moieties per cG250 molecule was determined as described by Hnatowich et al. The DTPA-cG250 conjugate was labeled with $^{111}$InCl$_3$ in a 0.1 mol/L ammonium acetate buffer of pH 5.4 for 30 min at room temperature. All radio-labeled cG250 preparations were purified by gel filtration on a PD-10 column eluted with phosphate buffered saline (PBS) supplemented with 0.5% bovine serum albumin (BSA). For all preparations, the amount of non-mAb-bound radiolabel was determined by instant thin-layer chromatography (ITLC) using ITLC silica gel strips, using 0.1 mol/L citrate buffer (pH 6.0) as the mobile phase.

**Biodistribution.** Tumor-bearing mice were injected intravenously with 3.7 MBq (0.1 mCi) $^{111}$In-DTPA-cG250 diluted in PBS + 0.5% BSA. All mice received a protein dose of 3-5 μg cG250 (volume, 200 μL/mouse). Mice were euthanized at 3 d after injection, and the tumor and normal tissues (blood, muscle, lung, spleen, kidney, liver, and small intestines without contents) were dissected, weighed, and counted in a γ-counter. To correct for radioactive decay, injection standards were counted simultaneously. The activity in samples was expressed as percentage of injected dose per gram tissue (%ID/g).

**Immunohistochemical staining.** PIMO (80 mg/kg) and the perfusion marker Hoechst 33342 (15 mg/kg) were injected 1 h and immediately prior to euthanizing the animals, respectively. From the frozen tumors, 5 μm sections were cut, mounted on poly-L-lysine coated slides and stored at -80 °C. After thawing and fixation, slides were scanned for the fluorescent signal of Hoechst 33342 before they were stained for CA-IX, PIMO and vessels. Between all consecutive steps of the staining, sections were rinsed three times for 5 min in PBS and all antibodies were diluted in primary antibody diluent (PAD). Sections were incubated overnight at room temperature with biotinylated mouse anti-CA9 (G250) antibody (E. Oosterwijk, Department of Urology, Radboud University Nijmegen Medical Centre) diluted 1:150. Then sections were incubated with mouse
anti-biotinCy3 diluted 1:400 for 30 min at 37 °C. This was followed by incubation with Fab fragment donkey anti-mouse IgG diluted 1:50 and rabbit anti-pimonidazole (J.A. Raleigh) diluted 1:1000 for 60 min at room temperature. Then sections were incubated with 9F1 (rat monoclonal to mouse endothelium, Department of Pathology, Radboud University Nijmegen Medical Centre) undiluted for 45 min at room temperature. Finally, sections were incubated with chicken anti-rat-Alexa647 diluted 1:200 and donkey anti-rabbit-Alexa488 diluted 1:400 for 60 min at 37 °C. Sections were mounted in Fluorostab.

Image acquisition and analysis. All tumor sections were analyzed using a digital image analysis system as described previously 22. After scanning whole tissue sections, grey scale images for vessels, perfusion (Hoechst), CA-IX and PIMO were obtained and subsequently converted into binary images. Thresholds for the fluorescent signals were interactively set above the background staining for each individual marker. Binary images were used to calculate the fractions of the tumor area positive for CA-IX (fCA-IX) and PIMO (fPIMO) relative to the total tumor area, the number of vessels and the proportion of perfused vessels. Areas of necrosis were excluded.

Statistics. Statistical analyses were performed on a Macintosh computer using the GraphPad Prism 4.0a software package (La Jolla, CA). Linear regression analysis was used to assess correlations between the different parameters and a p-value ≤ 0.05 was considered significant.

3.3 Results

In this study, three human head and neck tumor lines xenografted in nude mice were used, two squamous cell carcinomas (SCCNij3 and SCCNij59) and one mucoepidermoid carcinoma (MEC82). As illustrated in Figure 3.1, SCCNij59 showed peri-necrotic staining for PIMO (green) but no CA-IX expression. The SCCNij3 line showed areas of necrosis with PIMO and CA-IX (red) mainly expressed in areas around necrosis and areas of overlap between both markers (yellow), although generally, CA-IX was found at greater distance from vessels than PIMO. MEC82 expressed CA-IX abundantly and the staining pattern differed strongly from SCCNij3. PIMO was localized in the peri-necrotic regions and there was also overlap with CA-IX, but, in contrast to SCCNij3, CA-IX was already up-regulated closer to blood vessels than the PIMO staining.

3.3.1 Immunohistochemistry CA-IX, PIMO and vessels

Table 3.1 shows the results of the quantitative analysis of G250 concentration, fCA-IX, fPIMO and fraction of perfused vessels in the three tumor lines. SCCNij3 and MEC82 are relatively hypoxic tumors with high PIMO positive fractions. However, of these two
Chapter 3

Figure 3.1  Immunofluorescent images of SCCNij59 (A, B shows detail), SCCNij3 (C, D shows detail) and MEC82 (E, F shows detail) showing vessels (lightblue), pimonidazole binding (green) and CA-IX expression (red). Yellow represents areas of overlap between pimonidazole and CA-IX. Necrotic areas (N) were excluded from the analysis. All images were scanned at 100 x magnification, panels on left show complete tumor sections reduced in size.
tumors, MEC82 demonstrated significantly more CA-IX expression and good correlation
with PIMO staining \((p<0.0001, r^2=0.92, \text{Fig }3.2B)\). Expression of CA-IX was low in
SCCNij3 with, partly due to these low fCA-IX values, only a weak, non-significant
correlation with fPIMO (Fig 3.2A). SCCNij59 is a better oxygenated tumor and does not
express CA-IX. The fraction of perfused vessels was highest in MEC82 and lowest in
SCCNij3, but there was a large variability between tumors of the same tumor line.

Table 3.1  
Biodistribution, fCA-IX, fPIMO and fraction of perfused vessels in three xenograft tumor
lines. Values are mean ± 1 SD (range)

<table>
<thead>
<tr>
<th></th>
<th>Biodistribution</th>
<th>fCA-IX</th>
<th>fPIMO</th>
<th>Fraction vessels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(%ID/g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCCNij3</td>
<td>25.2 ± 6.2</td>
<td>0.02 ± 0.02</td>
<td>0.16 ± 0.1</td>
<td>0.56 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>(16.4 – 40.1)</td>
<td>(0-0.07)</td>
<td>(0.01 – 0.37)</td>
<td>(0.21 – 0.98)</td>
</tr>
<tr>
<td>SCCNij59</td>
<td>9.11 ± 3.3</td>
<td>0</td>
<td>0.03 ± 0.03</td>
<td>0.79 ± 0.24</td>
</tr>
<tr>
<td></td>
<td>(6.1 – 14.6)</td>
<td></td>
<td>(0 – 0.06)</td>
<td>(0.31 – 0.95)</td>
</tr>
<tr>
<td>MEC82</td>
<td>17.1 ± 6.1</td>
<td>0.10 ± 0.06</td>
<td>0.15 ± 0.08</td>
<td>0.88 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>(7.6 – 26.3)</td>
<td>(0-0.19)</td>
<td>(0-0.26)</td>
<td>(0.59 – 0.95)</td>
</tr>
</tbody>
</table>

3.3.2 G250 concentration versus CA-IX and PIMO immunohistochemistry
The two hypoxic tumors (SCCNij3 and MEC82) showed higher uptake of \(^{111}\text{In}\)-labeled
G250 relative to the well-oxygenated tumor (SCCNij59). The latter was CA-IX negative
as determined immunohistochemically. SCCNij3 and MEC82 showed reverse
associations between fCA-IX and G250 biodistribution. SCCNij3 demonstrated the
highest G250 uptake levels but low fCA-IX values, whereas MEC82 had the highest
fCA-IX values but lower G250 biodistribution levels. Correlations between fCA-IX or
fPIMO and G250 biodistribution within tumor lines were weak or absent (Fig 3.3).

Figure 3.2  
Scatterplots comparing fCA-IX versus fPIMO in 26 tumors of the SCCNij3 line and 12 tumors
of the MEC82 line. Linear best fit is shown.
3.4 Discussion

Carbonic anhydrase IX (CA-IX) is one of the most promising endogenous hypoxia-related cell markers known at present. Detectable by immunohistochemistry in biopsies, quantification of CA-IX expression by injection of the radio-labeled antibody could potentially allow non-invasive determination of CA-IX expression by PET or SPECT imaging.

However, comparisons of the expression and microregional distribution of CA-IX and the exogenous hypoxic cell markers such as PIMO did not show straightforward correlations. In the present study three human head and neck tumor lines were examined, two of which express CA-IX. Staining patterns for CA-IX and PIMO strongly differed between the two tumor lines. This is in agreement with previous findings by other groups. Lal et al. found similar expression patterns for CA-IX and PIMO in biopsy sections of a human oropharyngeal squamous cell carcinoma 13. In accordance with the findings in one of the tumor lines in the present study (MEC82), Olive et al. and Kaanders et al. demonstrated in carcinomas of the uterine cervix and head and neck that CA-IX staining was mainly observed at shorter distances from the blood vessels than PIMO staining 3,12. This indicates that CA-IX expression identifies areas with low as
well as intermediate tissue oxygenation status. In contrast, in skin and bladder carcinoma Wykoff et al. found that CA-IX staining was more dominant in peri-necrotic regions at greater distance to the vessels than PIMO staining. The peri-necrotic pattern of CA-IX is also found in SCCNij3 in the present study. Thus, the difference in relationship between PIMO staining and CA-IX expression is not simply related to tumor origin and histology, but also to the function of CA-IX in pH-maintenance in different tissues. The present study clearly shows large differences in CA-IX staining patterns even within one tumor entity. This indicates that the endogenous hypoxia-related marker CA-IX is not a straightforward surrogate for the exogenous hypoxic cell marker PIMO, but provides additional information possibly related to its function in pH-homeostasis.

In the two tumor lines of the present study that express CA-IX, only weak correlations were established between the two methods used to measure CA-IX. A possible reason for this could be the large interval of 3 days between injection of 111In-G250 and PIMO, the latter 1 h later followed by removal and rapid freezing of the tumors. In a recent publication, the half-life of hypoxic cells in the MEC82 line was reported to be 23 h, much shorter than for the SCCNij3 line (49 h). This implies that after a gap of 3 days, most of the hypoxic cells labeled by 111In-G250 have died, while in the SCCNij3 tumors close to half the number of labeled hypoxic cells is still present. This is further complicated by different CA-IX expression patterns between the tumor lines as discussed previously. MEC82 up-regulates CA-IX already at intermediate oxygenation levels, whereas in SCCNij3 up-regulation occurs only under severe hypoxia. Finally, the presence of tumor necrosis may contribute to the discordant results. With the immunohistochemical assessment, necrotic areas are excluded from analysis, but this is not possible with the 111In-G250 uptake assay, where activity in the total tumor volume is measured. It is unclear how fast biodegradation and washout of labeled antibodies occurs after tumor cell necrosis. Tumor blood perfusion and blood volume, vascular permeability and interstitial pressure, which differ between tumor lines, may play a role in this process as well. It is likely that labeled antibodies will remain detectable for some time in necrosis. Some suggestion for this is found in Figure 3.1, which demonstrates red fluorescent signal in necrotic areas. Yet it remains unclear whether this truly represents fluorescence-labeled antibody or merely aspecific staining.

It can be concluded from this preliminary set of data, with varying results on CA-IX and PIMO correlation, that CA-IX cannot be regarded as surrogate for the nitroimidazole based hypoxic cell markers such as pimonidazole. Furthermore, different methods of CA-IX measurement are not directly comparable and need further evaluation.
3.5 References


Correlation of $^{18}$FMISO autoradiography and pimonidazole immunohistochemistry in human head and neck carcinoma xenografts

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Abstract

Purpose. Tumor cell hypoxia is a common feature in solid tumors adversely affecting radiosensitivity and chemosensitivity in head and neck squamous cell carcinomas. Positron-emission tomography (PET) using the tracer $^{18}$Fluoromisonidazole ($^{18}$FMISO) is most frequently used for non-invasive evaluation of hypoxia in human tumors. A series of ten human head and neck xenograft tumor lines was used to validate $^{18}$FMISO as hypoxia marker at the microregional level.

Material and Methods. Autoradiography after injection of $^{18}$FMISO was compared with immunohistochemical staining for the hypoxic cell marker pimonidazole in the same tumor sections of ten different human head and neck xenograft tumor lines. The methods were compared, first qualitatively considering the microarchitecture, second by obtaining a pixel-by-pixel correlation of both markers at the microregional level, third by measuring the signal intensity of both images, and forth by calculating the hypoxic fractions by pimonidazole labeling.

Results. The pattern of $^{18}$FMISO signal was dependent on the distribution of hypoxia at the microregional level. The comparison of $^{18}$FMISO autoradiography and pimonidazole immunohistochemistry by pixel-by-pixel analysis revealed moderate correlations. In five tumor lines, a significant correlation between the mean $^{18}$FMISO and pimonidazole signal intensity was found (range $r^2=0.91$ to $r^2=0.99$). Comparison of the tumor lines with respect to the microregional distribution pattern of hypoxia revealed that the correlation between the mean signal intensities strongly depended on the microarchitecture. Overall, a weak but significant correlation between hypoxic fractions based on pimonidazole labeling and the mean $^{18}$FMISO signal intensity was observed ($r^2=0.18$, $p=0.02$). For the three tumor models with a ribbon-like microregional distribution pattern of hypoxia, the correlation between the hypoxic fraction and the mean $^{18}$FMISO signal intensity was much stronger and more significant ($r^2=0.73$, $p<0.001$) than for the tumors with a more homogeneous, patchy, microregional distribution pattern of hypoxia.

Conclusion. Different patterns of $^{18}$FMISO accumulation dependent on the underlying microregional distribution of hypoxia were found in ten head and neck xenograft tumors. A weak albeit significant correlation was found between the mean $^{18}$FMISO signal intensity and the hypoxic fraction of the tumors. In larger clinical tumors, $^{18}$FMISO-PET provides information on the tumor oxygenation status on a global level facilitating dose painting in radiation treatment planning. However, caution must be taken when studying small tumor subvolumes as accumulation of the tracer depends on the presence of hypoxia and on the tumor microarchitecture.
Figure 4.1  Left column: \(^{18}\)FMISO autoradiography images (A: SCCNij59, D: SCCNij153, G: SCCNij180); middle column: pseudo-colored grey-value images of SCCNij59 (B; ribbon-like hypoxia), SCCNij153 (E; mixed pattern of hypoxia) and SCCNij180 (H; patchy hypoxia) showing pimonidazole staining (green) and blood vessels (red); right column: detail of the pseudo-colored grey-value image at higher magnification, the area of origin is highlighted in the middle column (C: SCCNij59, F: SCCNij153, I: SCCNij180).
4.1 Introduction

Tumor cell hypoxia is a common feature in various solid human tumors, especially in human squamous cell carcinomas of the uterine cervix and the head and neck region. In these tumors, hypoxia is associated with a poor response to radiotherapy and cytotoxic agents, leading to worse treatment outcome and prognosis 1-5.

Treatment modifications counteracting the adverse effects of tumor cell hypoxia have been developed and applied in preclinical and clinical trials leading to better loco-regional tumor control and improved survival 6,7. To counteract hypoxia-associated radioresistance, hyperoxic gas breathing under normal or hyperbaric conditions, vasoactive drugs and hypoxic cell radiosensitizers have been used clinically. However, these treatment modifications are often accompanied by increased toxicity and morbidity. Furthermore, it is clear that not all patients benefit from these treatment intensifications. Patients with non-hypoxic tumors will not gain by hypoxia modulation, while still experiencing increased toxicity. Therefore, patient selection for these intensified treatment modalities is compulsory.

In accessible human tumors, e.g., tumors of the uterine cervix or metastatically involved lymph nodes of head and neck tumors, the invasive oxygen-sensitive Eppendorf histograph needle electrode can be used for measurement of the oxygen partial pressure (pO$_2$) 3,5,8-10. Alternatively, hypoxia can be studied after the intravenous (i.v.) injection of exogenous bio-reductive hypoxic cell markers such as EF5 and pimonidazole 11,12. These markers can be visualized with immunohistochemical techniques in biopsies or in tumor resection specimen. At pO$_2$ levels below 10 mmHg, the bio-reduction of these 2-nitroimidazoles is strongly increased 12,13. The predictive value of hypoxia detected by pimonidazole for treatment outcome was shown in advanced head and neck cancer 14.

A promising non-invasive imaging modality for detecting hypoxia is positron-emission tomography (PET) with radio-labeled tracers. PET enables functional imaging of the entire tumor mass and affected lymph nodes prior to treatment and might be used for improved target volume definition and delineation in radiotherapy, patient selection for treatment modification or for monitoring treatment efficacy during a course of radiotherapy or chemotherapy.

Currently, the most frequently used PET-tracer for detecting hypoxia in the clinical setting is $^{18}$F-fluoromisonidazole ($^{18}$FMISO) 15-21. The clinical relevance of $^{18}$FMISO-PET has recently been demonstrated in several patient studies 22-24; $^{18}$FMISO uptake was correlated with locoregional failure and outcome after therapy. Until present, however, the use of $^{18}$FMISO to image hypoxia has only been validated to a very limited extent. Therefore, knowledge about the performance of $^{18}$FMISO for
detection of intratumoral and intertumoral differences in the quantity and distribution of hypoxia is limited. In the present study, ten human head and neck xenograft tumors were investigated with different microregional distribution patterns of hypoxia: patchy, elongated ribbon-like and mixed. Intratumoral \(^{18}\)FMISO distribution as determined by autoradiography was correlated with immunohistochemical detection of pimonidazole with specific emphasis on marker distribution and signal intensity relative to the underlying patterns of hypoxia.

### 4.2 Material and Methods

**Animals and tumor models.** Nine human head and neck squamous cell carcinoma xenograft tumor lines (SCCNij59, SCCNij68, SCCNij86, SCCNij153, SCCNij154, SCCNij167, SCCNij180, SCCNij185 and SCCNij202) and one mucoepidermoid carcinoma xenograft line (MEC82) were used for comparison of the distribution of \(^{18}\)FMISO and pimonidazole. All tumor lines were established in-house from tumor biopsies of head and neck cancer patients. Viable 1 mm\(^3\) tumor samples were implanted subcutaneously in the abdominal flank of nu/nu BALB/c athymic mice and tumors were used for the experiments at a diameter of 8 - 9 mm. All mice were kept in accordance with institutional guidelines and the Animal Welfare Committee of the Radboud University Nijmegen approved all experiments.

**\(^{18}\)FMISO synthesis.** \(^{18}\)FMISO was synthesized according to the method described by Lim and Berridge. The 1-(2’-nitro-1’-imidazolyl)-2-O-tetrahydropyranyl-3-O-toluenesulfonyl-propanediol (NITTP) precursor was obtained from ABX GmbH (Radeberg, Germany) and \(^{18}\)Fluoride was obtained from BV Cyclotron VU (Amsterdam, The Netherlands). Radiosynthesis was performed in an automated synthesizer (Synchrom R&D, Raytest GmbH, Straubenhardt, Germany). Radiochemical yield (decay corrected) was always higher than 40% and radiochemical purity was always higher than 98%. Specific activity at the end of synthesis was always higher than 50,000 GBq/mmol.

**Experimental setup.** Tumor bearing mice were injected i.v. with 30 MBq \(^{18}\)FMISO in 0.2 mL. After 5 min, pimonidazole (1-[(2-hydroxy-3-piperidinyl) propyl]-2-nitroimidazole hydrochloride; Natural Pharmacia International, Research Triangle Park, NC) was injected intraperitoneally at a dose of 80 mg/kg. Mice from all ten xenograft lines were sacrificed by CO\(_2\)-asphyxiation one hour after the administration of \(^{18}\)FMISO. In order to analyze tracer distribution over time, additional mice bearing the SCCNij59 and MEC82 xenograft tumors (two tumors per line) were killed four hours after the injection. Immediately after killing the mice, tumors were dissected and snap frozen in
liquid nitrogen. Within 30 min, frozen tumor sections of 5 μm thickness were cut at the largest diameter and used for autoradiography of the 18F signal.

**Autoradiography.** Tumor sections were exposed to a phosphor imaging screen overnight. The screen was scanned in a phosphor imager system (Molecular Imager GS363, Bio-Rad Laboratories, Hercules, CA) at a pixel size of 100 μm × 100 μm (dynamic range: 4095 grey-values). Images were processed with Quantity One software (version 4.5.2, Bio-Rad Laboratories, Hercules, CA). The same tumor sections were then used for immunohistochemical staining and analysis of pimonidazole labeling.

**Immunohistochemical staining for hypoxia.** Between all consecutive steps of the staining procedure, the sections were rinsed three times for 5 min in 0.1 M phosphate-buffer saline (PBS, Klinipath, Duiven, The Netherlands), pH 7.4. The sections were first fixed in cold acetone (4 °C) for 10 min. After re-hydration of the tissue sections in PBS during 20 min, they were incubated with rabbit anti-pimonidazole (gift J.A. Raleigh, Department of Radiation Oncology, University of North Carolina School of Medicine, Chapel Hill, NC) diluted 1:1000 in primary antibody diluent (PAD, Abcam, Cambridge, UK) for 30 min. Sections were then incubated with donkey anti-rabbit-Alexa488 (Molecular Probes, Leiden, The Netherlands) diluted 1:400 in PBS for 30 min. Finally, sections were mounted with Fluorostab (Organon, Boxtel, The Netherlands).

**Immunohistochemical image acquisition.** All tumor sections were analyzed using a digital image analysis system as described previously. Grey-value images of pimonidazole were obtained after scanning whole tumor sections at a pixel size of 2.5 μm × 2.5 μm (dynamic range: 4095 grey-values). Thresholds for segmentation of the fluorescent signals were interactively set above the background level including all pimonidazole-positive tumor areas. Binary images were used to calculate the hypoxic fraction (HF; tumor area positive for pimonidazole relative to the total viable tumor area). Areas of necrosis were excluded from the analysis.

Analysis on co-localization, mean signal intensity and hypoxic fraction: The autoradiography and immunohistochemistry images were manually co-registered in IPLab (Scanalytics Inc., Fairfax, VA) and the pixel size of the latter was rescaled to match the pixel size of the phosphor imager. The images were then analyzed with in-house developed software that was implemented in IDL 6.0 (Research Systems, Boulder, CO), restricting the analysis to the tumor area and excluding areas of necrosis by drawing masks.

Pixel-by-pixel analysis on the grey-value images was performed for co-localization analysis. Scatter plots were created and subsequently a linear regression was performed. Furthermore, the mean signal intensities of both imaging modalities were compared using a linear regression. Finally, the hypoxic fraction obtained after
segmentation of the pimonidazole signal was correlated with the mean signal intensities of $^{18}$FMISO autoradiography using linear regression.

**Statistical analysis.** Linear regression analyses were performed using GraphPad Prism for Macintosh, version 4.0a (La Jolla, CA). The $p$-value, $r$ and $r^2$ were calculated. A $p$-value $\leq 0.05$ was considered statistically significant.

### 4.3 Results

#### 4.3.1 $^{18}$FMISO autoradiography and pimonidazole staining

Images after $^{18}$FMISO autoradiography, the corresponding pseudo-colored grey-value images of pimonidazole staining and details of the tumor sections at larger magnification were obtained (Fig. 4.1). Three of the ten human head and neck tumor lines studied showed a ribbon-like pattern of hypoxia detectable after immunohistochemical staining for pimonidazole (e.g., SCCNi59, Fig. 4.1B-C) 29. The corresponding autoradiography images showed a similar distribution of $^{18}$FMISO, with a high accumulation in pimonidazole positive and a low signal in pimonidazole negative areas. Six tumor lines showed a mixed pattern of hypoxia with ribbon-like and patchy components by immunohistochemistry (e.g., SCCNi153, Fig. 4.1E-F) 29. Co-localization of $^{18}$FMISO and pimonidazole was dependent on the underlying microregional distribution pattern of hypoxia. A clear correlation was only found in tumors with a ribbon-like pimonidazole distribution pattern. In tumors with a patchy distribution pattern, such as SCCNi180, accumulation of $^{18}$FMISO as detected by autoradiography occurred throughout the tumor section (Fig. 4.1H-I).

#### 4.3.2 Pixel-by-pixel analysis of $^{18}$FMISO autoradiography and PIMO immunohistochemistry grey-value images

Amongst the ten head and neck xenograft lines harvested one hour after tracer administration, a range of correlation coefficients after pixel-by-pixel grey-value analysis was found (Table 4.1). The underlying hypoxic pattern at the microregional level (e.g., ribbon-like versus patchy) did not influence the degree of correlation of the two imaging modalities. In accordance with the time interval frequently used in human studies, additional tumors originating from two lines (SCCNi59 and MEC82, two tumors per xenograft line) were studied 4 hours after $^{18}$FMISO injection. No improvement in correlation of the imaging modalities was observed (data not shown).

#### 4.3.3 Mean signal intensity of $^{18}$FMISO and pimonidazole

When analyzing all tumors together - irrespective of the xenograft line and thus underlying microarchitecture - no correlation between the mean $^{18}$FMISO and mean pimonidazole signal intensity was found (Fig. 4.2A). However, as the microarchitecture
might influence the degree of correlation, further analysis was performed taking the underlying hypoxic patterns into account. For the tumors with a ribbon-like distribution of hypoxia, the correlation of the mean signal intensities of both tracers was strong and statistically significant (SCCNij59: $r^2=0.99$, $p=0.05$; MEC82: $r^2=0.93$, $p=0.04$ and SCCNij167: $r^2=0.98$, $p=0.01$; Fig. 4.2B). Of the remaining seven xenograft tumor lines with a mixed or patchy pattern of hypoxia, only two with a mixed pattern of hypoxia showed a statistically significant correlation between the mean $^{18}$FMISO and pimonidazole signal intensities (SCCNij86: $r^2=0.93$, $p=0.03$ and SCCNij153: $r^2=0.91$, $p=0.05$; Fig. 4.2C).

### 4.3.4 Hypoxic fraction versus mean $^{18}$FMISO and pimonidazole signal intensity

A weak but statistically significant correlation between the hypoxic fraction based on segmentation of the pimonidazole signal and the mean $^{18}$FMISO signal intensity was found when all xenograft tumors were analyzed together ($r^2=0.18$, $p=0.02$; Fig. 4.3). For the three tumor lines with a ribbon-like hypoxia pattern (SCCNij59, MEC82 and SCCNij167), the correlation between the hypoxic fraction and the mean $^{18}$FMISO signal intensity was much stronger and more significant ($r^2=0.73$, $p<0.001$).

### Table 4.1

<table>
<thead>
<tr>
<th>Xenograft line</th>
<th>Number of tumors studied</th>
<th>Regression coefficient $r$</th>
<th>mean</th>
<th>SEM</th>
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<tr>
<td>SCCNij59</td>
<td>3</td>
<td>0.09</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>SCCNij68</td>
<td>4</td>
<td>0.11</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>MEC82</td>
<td>4</td>
<td>0.12</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>SCCNij86</td>
<td>4</td>
<td>0.06</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>SCCNij153</td>
<td>4</td>
<td>0.19</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>SCCNij154</td>
<td>5</td>
<td>0.09</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>SCCNij167</td>
<td>4</td>
<td>0.05</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>SCCNij180</td>
<td>5</td>
<td>0.04</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>SCCNij185</td>
<td>4</td>
<td>0.06</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>SCCNij202</td>
<td>3</td>
<td>0.19</td>
<td>0.06</td>
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</tr>
</tbody>
</table>
Figure 4.2  Mean $^{18}$FMISO versus mean pimonidazole signal intensity of all xenografts tumor lines studied (A) and of the tumors with a ribbon-like (B) or patchy and mixed (C) pattern of hypoxia.
### 4.4 Discussion

Tumor cell hypoxia is a tumor characteristic responsible for poor treatment outcome and prognosis, especially in squamous cell carcinoma of the head and neck. Various treatment modifications aiming at reducing tumor hypoxia have been developed. Most of these treatment intensifications are at the cost of increased toxicity, necessitating patient selection. The assessment of hypoxia by endogenous (e.g., Glut-1 and -3, CA-IX) or exogenous hypoxic cell markers (e.g., EF5 and pimonidazole) enables pre-treatment assessment of tumor hypoxia. Non-invasive imaging using PET provides biological and geometrical information on the primary tumor and on potentially metastatic lymph nodes or distant metastases. This information is required for patient selection prior to treatment initiation and for radiation treatment planning. Furthermore, these techniques make it possible to monitor tumor response during treatment, eventually allowing to adjust the treatment based on tumor response characteristics.

Clinical and preclinical studies published on $^{18}$FMISO have only been performed on small cohorts of patients and on a limited number of experimental tumors and tumor cell lines. Therefore, little is known about the influence of the microarchitecture on the $^{18}$FMISO distribution and the correlation with immunohistochemical visualization of 2-nitroimidazole derivates. The aim of this study was to validate $^{18}$FMISO autoradiography versus pimonidazole immunohistochemistry in ten human head and neck xenograft models, both qualitatively and quantitatively, and furthermore to analyze whether tumor microenvironmental characteristics affect $^{18}$FMISO distribution. In a series of human xenografts originating from squamous cell carcinomas of the head and neck, Ljungkvist et al. described three different patterns of distribution of hypoxia: patchy, elongated ribbon-like and mixed. The patchy hypoxic pattern is characterized by larger areas of chronic hypoxia with gradients of pimonidazole binding. The ribbon-
like pattern displays narrower zones of hypoxia with more intense pimonidazole binding and at further distance from the vasculature necrosis. In tumors with ribbon-like patterns of hypoxia, a strong $^{18}$FMISO accumulation was detected in the viable pimonidazole positive areas with no signal in necrotic or normoxic regions. The tumors with a patchy or mixed pattern of pimonidazole binding with little or no necrotic regions demonstrated a more diffuse $^{18}$FMISO signal throughout the tumor.

These heterogeneous patterns of accumulation were also seen in studies by others using $^{18}$FMISO or $^{64}$CuATSM $^{21,25,27}$. Wyss et al. analyzed ten human xenografts originating from various tumor types using NanoPET imaging $^{21}$. They described a category of tumors with a high-count density in the rim of the tumor surrounding a central part of low radioactivity and another category with a spotty distribution of $^{18}$FMISO, indicating a heterogeneous pattern of hypoxia. This finding was supported by Allemann et al., who imaged seven cats with vaccine-associated sarcoma and found tumors with high focal $^{18}$FMISO uptake and others with a more patchy distribution of the hypoxia PET-tracer $^{25}$. Using $^{64}$CuATSM as PET tracer, Tanaka et al. performed autoradiography and immunohistochemistry in various xenografts and found $^{64}$CuATSM accumulation to be present at the edge of the tumors with no accumulation in the necrotic areas $^{27}$. Two tumor characteristics at the microregional level may explain these heterogeneous patterns of accumulation. As mentioned above, tumors with a ribbon-like pattern of hypoxia receive their blood-supply from vascular sheets as compared to patchy tumors with a central vessel being responsible for the blood-supply. These vascular structures are responsible for the PET tracer influx, but also for the wash-out of the tracer. One could therefore hypothesize that the vascular sheets found in ribbon-like tumors are more effective in facilitating tracer influx and wash-out. Furthermore, in the tumor lines with a ribbon-like hypoxia pattern, hypoxia may be more severe compared to the patchy tumors, resulting in an intense pimonidazole and $^{18}$FMISO binding. As a result of this, the hypoxic pattern of ribbon-like tumors imaged by $^{18}$FMISO autoradiography is likely to be more distinct compared to the patchy tumors with more extensive areas with a distribution of hypoxia.

In the current study, the microregional distribution and spatial correlation between $^{18}$FMISO and pimonidazole accumulation was assessed quantitatively by pixel-by-pixel analysis. Results obtained by these analyses varied between different xenograft lines, but were not influenced by the underlying hypoxic pattern $^{20}$. Yuan et al. studied three histologically different human xenografts using microPET and autoradiography, and found the correlation between $^{64}$CuATSM autoradiography and EF5 immunohistochemistry to differ substantially between tumor lines from $r^2=0.05$ to $r^2=0.78$ $^{28}$. In two out of the three lines studied, the regression coefficients were high compared to our study. However, the scatter plots show that pixels outside the tumor area and presumably also in necrotic areas were included in the pixel-by-pixel analysis. In our study, signal intensities outside the tumor and in necrotic areas were excluded.
from the analysis resulting in lower regression coefficients. Another explanation for the weak correlation found in several of our tumor lines could be the difference in binding characteristics of pimonidazole and $^{18}$FMISO. For the pimonidazole staining, an antibody that is raised against the reduced pimonidazole adducts is used and therefore, extracellular (non-reduced) pimonidazole is not detected. In contrast to this, the signal of the autoradiography images comprises both, bound and reduced, and unbound non-reduced $^{18}$FMISO. Finally, manual co-registration of the images may introduce mismatches that influence the results of the pixel-by-pixel analysis.

A strong and significant positive correlation between mean signal intensity of $^{18}$FMISO and pimonidazole was found in five of the ten xenograft lines studied. Remarkably, within these five tumors, all tumors with a ribbon-like pattern of hypoxia are presented. In these specific tumors, the influx, wash-out, accumulation and reduction of both tracers must be very similar resulting in a strong correlation of the mean signal intensities of both modalities.

The correlation of the hypoxic fraction, obtained after segmentation of the pimonidazole signal, with the mean $^{18}$FMISO signal intensity showed a significant albeit weak correlation when including all ten xenografts lines in the analysis. Not surprisingly, when only including the tumors with a ribbon-like pattern of hypoxia, the correlation of mean $^{18}$FMISO signal intensity and the hypoxic fraction improved. These findings are in agreement with results by Hoebers et al. on ten head and neck cancer patients $^{32}$. They found a correlation between SPET scans using the novel radioactively labeled 2-nitro-imidazole hypoxic marker $^{99m}$Tc-BRU $^{59-21}$ and pimonidazole-positive areas (primary tumor: $r=0.73$, $p=0.016$; primary tumor + lymph nodes: $r=0.86$, $p<0.001$). Additionally, the correlation was found to vary between individual patients supporting our hypothesis on the influence of the microarchitecture. Also, these findings support the observation that correlation of different imaging modalities (and different tracers) may vary depending on the imaging methods used and are not necessarily interchangeable $^{33}$. However, more importantly, our results and those by Hoebers et al. show that the tumor oxygenation status macroscopically assessed by nuclear imaging techniques corresponds with the overall hypoxic fraction. This indicates that $^{18}$FMISO-PET can provide prognostic and predictive information on the overall oxygenation status of a tumor. The prognostic potential was shown by Thorwarth et al., who found a correlation between $^{18}$FMISO uptake and radiotherapy outcome in a series of 12 head and neck cancer patients $^{24}$. Rischin et al. showed the predictive potential of $^{18}$FMISO in 45 patients with advanced head and neck cancer $^{23}$. They performed pre-treatment and midtreatment $^{18}$FMISO-PET scans and found $^{18}$FMISO-PET to be predictive for the response to the hypoxic cytotoxin tirapazamine.
In contrast to these encouraging results on entire tumor volumes, the use of $^{18}$FMISO-PET for the identification of smaller hypoxic areas, for instance when considering boosting hypoxic tumor subvolumes with radiation therapy, probably depends on the microarchitectural organisation of the tumors.

### 4.5 Conclusions

Comparison of $^{18}$FMISO with pimonidazole in a variety of human head and neck xenograft tumors revealed different patterns of tracer accumulation depending on the tumor microarchitecture. A significant correlation was found between the mean $^{18}$FMISO signal intensity and the hypoxic fraction of the tumor assessed by pimonidazole staining. $^{18}$FMISO-PET may provide information on the global tumor oxygenation status, but is less reliable for studying tumor subvolumes.

### Acknowledgements

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4.6 References


Imaging hypoxia after oxygenation-modification: comparing $^{18}$FMISO autoradiography with pimonidazole immunohistochemistry in human xenograft tumors

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Abstract

Purpose. One of the major reasons of resistance to radiation therapy is tumor cell hypoxia. Visualization and quantification of the hypoxic cell marker pimonidazole has been shown to be a potential predictor for treatment outcome in advanced stage head and neck cancer. Positron emission tomography (PET) using $^{18}$F-labeled misonidazole ($^{18}$FMISO) is a non-invasive way of imaging tumor hypoxia. However, in contrast to pimonidazole, little is known about its value under varying levels of hypoxia induced by oxygenation modifying treatment. Aim of this study was to validate the PET-tracer $^{18}$FMISO against pimonidazole immunohistochemistry under different oxygenation conditions.

Material and Methods. A human head and neck squamous cell carcinoma (SCCNij3) and two human glioblastoma (E102 and E106) xenograft tumor lines were studied after injection of $^{18}$FMISO and pimonidazole. Control mice were compared with a second group breathing carbogen (95% O$_2$ and 5% CO$_2$) to reduce tumor hypoxia and a third group, where tumors were clamped to increase hypoxia. Tumor sections were analyzed overnight using a phosphor imaging system for autoradiography and consecutively, the same sections were stained immunohistochemically (IHC) for visualization of pimonidazole. A pixel-by-pixel analysis was used to compare $^{18}$FMISO with pimonidazole. Also, the hypoxic fraction obtained after segmentation of the pimonidazole signal, was related to the mean optical density of $^{18}$FMISO and pimonidazole.

Results. A moderate pixel-by-pixel correlation between $^{18}$FMISO autoradiography and pimonidazole IHC was found for the control tumors, after carbogen breathing and after clamping for SCCNij3. Only very weak correlations were found for the glioblastoma lines. Mean signal intensities for pimonidazole significantly decreased after carbogen breathing and increased after clamping in E102 and E106. Mean $^{18}$FMISO signal intensities increased significantly after clamping for both glioma xenograft tumor lines. A significant correlation between the hypoxic fractions obtained after segmentation of the pimonidazole signal and the mean $^{18}$FMISO signal intensities was found only for the glioblastoma xenograft tumor lines ($r^2$ ranging from 0.28 to 0.86).

Conclusions. $^{18}$FMISO autoradiography and pimonidazole immunohistochemistry can both be used to visualize treatment induced changes in tumor hypoxia. However, the response to these modifications differs widely between xenograft tumor lines. Therefore, clinical studies validating $^{18}$FMISO against pimonidazole immunohistochemical staining in different tumor entities are necessary.
5.1 Introduction

Hypoxia is present in most solid tumors due to impaired oxygen and nutrient supply to the tumor. Hypoxia is associated with an increased likelihood of locoregional recurrence and distant metastases. Furthermore, hypoxia decreases the response to radiation therapy and to several cytotoxic agents, leading to poor treatment outcome and prognosis. To overcome hypoxia-induced radioresistance, several treatment modifications have been developed, such as the use of hyperoxic gas breathing under normal or hyperbaric conditions, vasoactive drugs and hypoxic cell sensitizers. These modifications often lead to increased toxicity and morbidity for the patient. Furthermore, not all patients benefit from this treatment intensification. For instance, patients with non-hypoxic tumors will not gain by hyperoxic gas breathing while still experiencing the increase in side effects. Therefore, careful selection of patients for these intensified treatment modalities is necessary.

Traditionally, the oxygen partial pressure (pO$_2$) in accessible human tumors, e.g., the uterine cervix and lymph nodes, is measured using the invasive oxygen-sensitive Eppendorf histograph needle electrode. Hypoxia can also be detected by visualization of bio-reductive hypoxic cell markers in tumor sections. The two exogenous hypoxia markers approved for patient studies are EF5 and pimonidazole. Bio-reduction of these 2-nitroimidazoles strongly increases at pO$_2$ levels below 10 mmHg. Recently, the predictive value of hypoxia detected by pimonidazole for treatment outcome was shown in advanced stage head and neck cancer. The use of endogenous hypoxia-related markers, such as carbonic anhydrase IX (CA-IX) or glucose transporters (Glut-1 and -3), has been extensively investigated for this purpose. However, results thus far are conflicting.

Although these hypoxic cell markers are excellent tools for quantification of hypoxia at the microregional level, these assays cannot be used to monitor changes of tumor hypoxia over time. Promising non-invasive imaging modalities permitting repeated measurements include magnetic resonance imaging techniques and positron-emission tomography (PET) of radio-labeled bio-reductive markers. Currently, the clinically most widely used hypoxia PET-tracer is $^{18}$F-fluoromisonidazole ($^{18}$FMISO). However, $^{18}$FMISO cannot be validated as a hypoxia marker at microregional level as no antibody is available until present.

This study was designed to evaluate the potential usefulness of $^{18}$FMISO for the detection of changes in tumor hypoxia in three human xenograft tumor lines after carbogen (95% O$_2$, 5% CO$_2$) breathing and clamping and compare the results with the well established hypoxic cell marker pimonidazole.
5.2 Material and Methods

Animals and tumor models. The human head and neck squamous cell carcinoma xenograft tumor line SCCNij3, and the human glioblastoma tumor lines E102 and E106 were used for these experiments. Viable 1 mm³ tumor pieces were implanted subcutaneously in the abdominal flank of athymic BALB/C nu/nu mice and tumors were used for the experiments at a diameter of 8 - 9 mm. All mice were kept in accordance with institutional guidelines. All experiments were approved by the Animal Experiments Committee of the Radboud University Nijmegen.

18FMISO synthesis. 18FMISO was synthesized according to the method described by Lim and Berridge 21. The 1-(2’-nitro-1’-imidazolyl)-2-O-tetrahydropyranyl-3-O-toluenesulfonyl-propanediol (NITTP) precursor was obtained from ABX GmbH (Radeberg, Germany) and 18Fluoride was obtained from BV Cyclotron VU (Amsterdam, The Netherlands). Radiochemical yield (decay corrected) was always higher than 40% and radiochemical purity was always higher than 98%. Specific activity at end of synthesis was higher than 50,000 GBq/mmol.

Experimental setup. Mice were stratified into three groups of 5-6 animals, based on tumor size; one group served as control, the other groups were studied either under carbogen breathing or clamping. At the start of the experiment, the control animals were injected i.v. with 37 MBq of the hypoxia PET-tracer 18FMISO in 0.2 mL. After five minutes, the exogenous hypoxia marker pimonidazole hydrochloride (1-[(2-hydroxy-3-piperidinyl) propyl]-2-nitroimidazole hydrochloride; Natural Pharmacia International, Research Triangle Park, NC) was injected i.p. at a dose of 80 mg/kg. One hour after injection of 18FMISO the animals were killed by cervical dislocation. The mice in the carbogen group started breathing carbogen (95% O₂, 5% CO₂; Hoek Loos, Schiedam, The Netherlands) 5 min before injection of 18FMISO and continued until the animals were killed. In the clamping group, 30 min after administration of 18FMISO, tumors were clamped for 30 or 60 min until killing of the animals. Immediately after killing the mice, tumors were removed and snap frozen in liquid nitrogen. Within 30 min, frozen tumor sections of 5 µm thickness were cut from the central part of the tumor and sections were used to detect the 18FMISO signal on the phosphor imaging system (PPI).

Autoradiography. From each tumor, a central 5 µm section was cut and mounted on poly-L-lysine coated slides. Tumor sections were exposed to the phosphor imager overnight. The screen was scanned in a phosphor imager system ( Molecular Imager GS363, BioRad Laboratories, Hercules, CA) at a pixel size of 100 µm × 100 µm. Images were processed with Quantity One software (version 4.5.2, BioRad Laboratories, Hercules, CA). The same tumor section was then used for immunohistochemical staining and analysis of pimonidazole labeling.
**Immunohistochemical staining for hypoxia and blood vessel perfusion.** Between all consecutive steps of the staining procedure, the sections were rinsed three times for 5 min in 0.1 M phosphate-buffer saline (PBS, Klinipath, Duiven, The Netherlands), pH 7.4. The sections were first fixed in cold acetone (4 °C) for 10 min. After re-hydration of the tissue sections in PBS during 20 min, they were incubated with rabbit anti-pimonidazole (gift J.A. Raleigh) diluted 1:1000 in primary antibody diluent (PAD, Abcam, Cambridge, UK) for 30 min. Sections were then incubated with donkey anti-rabbit-Alexa488 (Molecular Probes, Leiden, The Netherlands) diluted 1:400 in PBS for 30 min. For vessel staining, sections were first incubated with undiluted 9F1 (rat monoclonal to endothelium; Department of Pathology, Radboud University Nijmegen Medical Centre, The Netherlands) for 30 min and then with goat anti-rat-TRITC (Jackson Immuno Research Laboratories Inc., West Grove, PA), diluted 1:200 in PBS for 30 min. Finally, sections were mounted on Fluorostab (Organon, Boxtel, The Netherlands).

**Co-localization analysis.** The autoradiography and immunohistochemistry images were manually co-registered in IPLab (Scanalytics Inc., Fairfax, VA) and the pixel size of the latter was rescaled to meet that of the autoradiography (pixel size: 100 μm × 100 μm). The images were then analyzed with in-house developed software that was implemented in IDL 6.0 (Research Systems, Boulder, CO). The rescaled and co-registered images were used to create scatter plots, which were fitted with a linear regression line. In addition, mean, median and standard deviation of the signal intensities were calculated.

**Statistical analysis.** Pixel-by-pixel correlations were calculated in IDL. For inter- and intratumoral correlation, mean, median and standard deviation were calculated by linear regression and by applying the Kruskal-Wallis test (GraphPad Prism for Macintosh, version 4.0a; La Jolla, CA). A p-value ≤ 0.05 was regarded statistically significant.

### 5.3 Results

#### 5.3.1 18FMISO autoradiography and pimonidazole staining

Figure 5.1 depicts examples of hypoxia visualized after pimonidazole immunohistochemistry, segmentation of the pimonidazole signal and 18FMISO autoradiography of the SCCNij3 and E106 tumors. In the SCCNij3 tumors, visualization of pimonidazole revealed a significant increase of the hypoxic fraction (obtained after segmentation of the grey-value image) after clamping and a decrease of the hypoxic fraction after carbogen breathing (Table 5.1). However, in this tumor no significant changes in the 18FMISO autoradiography images were observed with only a minimal response after carbogen breathing. In the E102 and E106 tumors, a reduction of the
hypoxic fraction in the carbogen group was found as well as an increase after clamping (Fig. 5.1 and Table 5.1). $^{18}$FMISO autoradiography after clamping showed an increase in signal intensity. After carbogen breathing only a minimal reduction of $^{18}$FMISO signal was observed.

5.3.2 Pixel-by-pixel analysis of autoradiography and immunohistochemistry

Figure 5.2 shows examples obtained after pixel-by-pixel analysis based on pixel intensity illustrating a moderate correlation between pimonidazole immunohistochemistry and $^{18}$FMISO autoradiography for SCCNij3. The mean regression coefficient ($r^2$) was 0.10 for the controls, 0.12 after carbogen breathing and 0.29 after clamping. For E102 the mean $r^2$ was 0.05 for the control, 0.06 for the carbogen breathing and -0.02 for the clamping group. Corresponding mean $r^2$-values for E106 were 0.14 for the controls, 0.07 after carbogen breathing and 0.03 after clamping.

<table>
<thead>
<tr>
<th>Table 5.1</th>
<th>Mean hypoxic fraction of xenograft tumor lines</th>
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<tr>
<td></td>
<td>Hypoxic fraction (SEM)</td>
</tr>
<tr>
<td></td>
<td>(tumor area positive for pimonidazole compared to total tumor area)</td>
</tr>
<tr>
<td>control</td>
<td>carbogen</td>
</tr>
<tr>
<td>SCCNij3</td>
<td>0.06 (0.03)</td>
</tr>
<tr>
<td>E102</td>
<td>0.03 (0.00)</td>
</tr>
<tr>
<td>E106</td>
<td>0.03 (0.00)</td>
</tr>
</tbody>
</table>

SEM = standard error of the mean, * = relative to control, n.s. = not significant

5.3.3 Mean signal intensity of $^{18}$FMISO and pimonidazole

Figure 5.3 illustrates the analysis of mean signal intensities of pimonidazole and $^{18}$FMISO. In contrast to the hypoxic fraction, which is obtained after segmentation of the pimonidazole image, the mean signal intensity of both pimonidazole and $^{18}$FMISO showed only a minor change after carbogen breathing. Only for E102, and to a lesser extent for E106, a significant reduction of the mean pimonidazole signal intensity was observed (Fig. 5.3). Clamping of the tumors resulted in a significant increase of the mean signal intensity of both pimonidazole and $^{18}$FMISO in all tumors, except in SCCNij3 in which the increase of $^{18}$FMISO signal by autoradiography after clamping was not statistically significant.

5.3.4 Hypoxic fraction (HF) versus signal intensity

Figure 5.4 shows hypoxic fraction versus mean signal intensity of pimonidazole and $^{18}$FMISO. For SCCNij3 (Fig. 5.4A), a significant but weak correlation between hypoxic fraction and mean pimonidazole signal intensity was found. For both, the E102 (Fig. 5.4B) and E106 (Fig. 5.4C) xenograft tumor lines, we found a statistically significant correlation between the HF and both the mean PIMO and $^{18}$FMISO signal intensity.
Figure 5.1  Pseudo-colored grey-value pimonidazole images (top row), images after segmentation of the pimonidazole signal (middle row) and $^{18}$FMISO autoradiography images (bottom row) of SCCNij3 and E106 for control tumors (a), after carbogen breathing (b) and clamping (c).
Figure 5.2  Examples of scatter plots obtained after pixel-by-pixel analysis of pimonidazole intensity and ^18FMISO intensity for the control (left panel), carbogen (middle panel) and clamping (right panel) group of SCCNi3 (top row), E102 (middle row) and E106 (bottom row) with the best linear fit. Regression coefficient $r^2$ is shown.
Figure 5.3  Mean signal intensities of pimonidazole and $^{18}$FMISO in the control, carbogen breathing and clamping groups. A $p<0.05$ is indicated by *, a $p<0.01$ by ** and a $p<0.001$ by ***, Kruskal-Wallis test was applied. Error bars represent standard deviation.
Chapter 5

Figure 5.4  Linear regression analysis comparing hypoxic fraction obtained after segmentation of the pimonidazole immunohistochemical staining with mean pimonidazole and $^{18}$FMISO signal intensities. P-values and $r^2$ are shown.

5.4 Discussion

A well-established method for quantification of hypoxia is the use of hypoxic cell markers such as pimonidazole. Immunohistochemical detection of pimonidazole in tissue sections has been shown to predict for prognosis in advanced stage head and neck cancer 11. A possible alternative to this method is PET imaging of the hypoxic tracer $^{18}$FMISO. In various solid tumors hypoxia was detected using $^{18}$FMISO-PET 16,18-20.
The advantage of imaging hypoxia with a $^{18}$FMISO-PET scan is that it can be used repeatedly on an almost daily basis due to the short half-life of the tracer (1.83 h). Another advantage is the possibility to visualize hypoxia in the whole tumor, albeit at a low resolution (5 mm). Aim of this study was to validate the hypoxia PET-tracer $^{18}$FMISO against pimonidazole immunohistochemistry under different oxygenation conditions. For this purpose, three human xenograft tumor lines were studied under ambient conditions and after carbogen breathing and clamping of the tumors.

Assessment of the microregional distribution of hypoxia was based on immunohistochemical staining of the bio-reductive hypoxia marker pimonidazole. Hypoxic fractions obtained after image analysis showed a decrease after carbogen breathing and an increase after clamping, the extent of the changes varied widely between the tumor lines.

In the present study, hypoxia detected by pimonidazole staining was quantified in two different ways: first after segmentation of the pimonidazole-based grey-value images and second based on intensity profiles of both $^{18}$FMISO and pimonidazole (Fig. 5.1). Previously, it was shown that the range of staining profiles of the nitroimidazole EF5 - detected by immunohistochemical staining techniques directly correlates with hypoxia levels in tumor sections 22. For visualization of pimonidazole we used a two-step staining technique that has the advantage of creating an optimal signal-to-noise ratio. The disadvantage of an indirect labeling technique is that the range of intensity values could be affected. This may explain the observation that correlations related to intensity profiles of pimonidazole and $^{18}$FMISO are only moderate. However, by optimizing the signal-to-noise ratio, segmentation of the grey-value images becomes more reliable. The result is that the hypoxic fraction is a more robust parameter for tumor hypoxia than that based on intensity profile analysis.

Dubois et al. compared $^{18}$FMISO microPET and autoradiography with immunohistochemistry (pimonidazole and CA-IX) under ambient conditions in a rat rhabdomyosarcoma model 23. A significant correlation between $^{18}$FMISO-PET and pimonidazole–positive volume was found. In that study, both volumes were based on segmentation of the microPET and staining images and not on intensity profiles. In our study, for two of the three xenograft tumor lines, a significant correlation was also found between hypoxic fractions and the mean $^{18}$FMISO signal intensity, which clearly shows the heterogeneity of tumors in response to carbogen breathing.

The pixel-by-pixel correlation between the mean signal intensity of pimonidazole and $^{18}$FMISO varied from very weak to moderate in the three xenograft tumor lines. This may be due to the reformatting of the pixel size of the immunohistochemical image to the pixel size of the phosphor imager. In this procedure, microregional differences in signal intensity are lost due to integration over a relatively large volume. Small inaccuracies after manual co-registration of both images may introduce mismatch between both imaging modalities resulting in a further decrease of the pixel-by-pixel
correlation. Finally, differences may also be tumor line dependent. The pixel-by-pixel correlation is stronger for tumors with larger patches of hypoxia and weaker if hypoxia is present in smaller areas throughout the tumor.

Clamping resulted in a significant increase of the mean signal intensity for both pimonidazole and $^{18}$FMISO relative to control tumors in the two glioblastoma xenograft lines. However, a significant reduction in mean signal intensity after carbogen breathing was only found for the mean pimonidazole signal intensity in these tumors and not for the mean $^{18}$FMISO signal intensity. This higher than expected activity of $^{18}$FMISO could be caused by intra-tumor trapping of unbound marker. Van der Sanden et al. described a decrease in tumor blood perfusion after carbogen breathing - measured by MR-imaging of gadolineum DTPA-uptake - in a glioblastoma xenograft tumor line. This decrease in perfusion may lead to an incomplete wash-out of $^{18}$FMISO resulting in relatively high values after carbogen breathing. Bentzen et al. found a significant reduction in $^{18}$FMISO PET defined hypoxic tumor volume in C3H mammary carcinoma after carbogen breathing. With this same tumor model, Grönroos et al. showed a decrease in $^{18}$FMISO signal intensity by autoradiography after carbogen breathing relative to the control tumors. These results indicate that the response to carbogen breathing likely depends on the tumor model studied and may potentially also be influenced by the site of tumor transplantation, such as orthotopic versus subcutaneous transplantation.

5.5 Conclusions

Pimonidazole labeling can be used to detect viable hypoxic cells at pO$_2$ levels below 10 mmHg. By visualizing pimonidazole, hypoxia can be studied with a high spatial resolution providing detailed information at the microscopic level. $^{18}$FMISO can be used as PET-tracer to study hypoxia at the global level in tumors in situ. In the present study, pimonidazole labeling was used for validation of $^{18}$FMISO autoradiography. This pimonidazole signal reflects only reduced and bound marker. The autoradiography signal may represent bio-reduced intracellularly bound as well as unbound extracellular radioactivity that is trapped in the tumor. Therefore, the results obtained by using PET-markers such as $^{18}$FMISO are susceptible to inaccuracies if insufficient time is allowed for biodistribution, bio-reduction and wash-out of unbound marker. Both imaging methods provide information on the level and spatial distribution of hypoxia; at the microscopic level by pimonidazole and at a more global level by $^{18}$FMISO.
Acknowledgements

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5.6 References


Chapter 6

Histopathological validation of $^{18}$FLT-PET in squamous cell carcinomas of the oral cavity

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Abstract

Introduction. Accelerated tumor cell repopulation is an important mechanism adversely affecting therapeutic outcome in head and neck cancer. Non-invasive assessment of a tumor’s proliferative state by positron emission tomography (PET) may provide a selection tool for customized treatment. 3'-deoxy-3'-18F-fluorothymidine (18FLT) is a PET tracer that is phosphorylated by thymidine kinase 1 (TK-1) and as such reflects cellular proliferation. Before the use of 18FLT-PET for tumor characterization is accepted and introduced into clinical studies, validation against tumor histology is mandatory. The aim of this study was to validate 18FLT-PET in squamous cell carcinomas of the oral cavity using immunohistochemical staining for the proliferation marker iododeoxyuridine (IdUrd) and for TK-1.

Materials and Methods. Seventeen patients with primary squamous cell carcinomas of the oral cavity underwent an 18FLT-PET-CT scan prior to surgery, and IdUrd was administered 20 min prior to tumor resection. 18FLT-PET-CT scans were segmented, and CT/PET volumes and PET signal intensities calculated (SUV\text{mean} and SUV\text{max}). Multiple paraffin-embedded tumor sections were immunohistochemically stained for IdUrd and TK-1. For IdUrd, labeling indices and optical densities were calculated and correlated with SUV\text{mean} and SUV\text{max}. TK-1 staining was visually and semi-quantitatively assessed.

Results. All primary tumors were identified with 18FLT-PET, but with a large range in tracer uptake (mean SUV\text{max} 5.9; range 2.2-5.2). Also, there was a large variability in IdUrd labeling indices (mean 0.09; range 0.01-0.29) and optical densities (mean 28.2; range 12.6-37.8). The IdUrd optical densities correlated significantly with SUV\text{mean} and SUV\text{max}, but the labeling index did not. In most tumors, TK-1 staining of varying intensity was present, but did neither correlate with IdUrd binding nor with 18FLT-uptake.

Conclusions. The current study demonstrated only a weak correlation between 18FLT-uptake and IdUrd staining intensity in oral cavity tumors. This may be explained by differences in biomarker characteristics, resolution, and quantification methods. The potential role of 18FLT-PET in clinical oncology remains to be further explored.
6.1 Introduction

Positron emission tomography (PET) with the glucose analogue 2-18F-fluoro-2-deoxy-D-glucose (18FDG) is accepted as a powerful, non-invasive metabolic imaging method suitable for diagnosis and staging of various types of cancer. Apart from high-energy expenditure, tumor cell repopulation is an important indicator of tumor aggressiveness and of its resistance to various types of treatment including radiotherapy. As a response to radiotherapy, squamous cell carcinomas of the head and neck show accelerated repopulation of clonogenic tumor cells during the course of treatment adversely affecting treatment outcome. Treatment regimes counteracting this effect have demonstrated to improve the outcome of head and neck cancer. Randomized trials have convincingly shown that a reduction of the overall treatment time by accelerated radiotherapy schedules increases the locoregional tumor control rate by about 10%. Targeting the signal transduction pathways that control tumor cell proliferation by inhibition of the epidermal growth factor receptor (EGFR) in combination with radiotherapy improves not only local tumor control, but also ultimate survival.

Non-invasive assays monitoring the proliferative activity of the tumor before and during therapy may assist in better patient selection for these intensified treatment strategies. Shields et al. introduced the thymidine analogue 3′-deoxy-3′-18F-fluorothymidine (18FLT) as PET tracer for visualization of proliferating (tumor) cells. 18FLT is transported via the membranous human nucleoside transporter 1, phosphorylated by the cytosolic thymidine kinase 1 (TK-1) enzyme and trapped intracellularly. During the late G1 and S phase of the cellular cycle, TK-1 activity increases by almost tenfold. As 18FLT trapping is related to TK-1 activity, it is in turn also linked to cellular proliferation.

Before treatment decisions can be based on these functional imaging modalities, it is of utmost importance that histological validation studies are performed. Quantitative 18FLT-PET data must be correlated with quantitative data of proliferation at the tissue and cellular level obtained by histological examination.

The aim of this study was to validate 18FLT-PET imaging by immunohistochemical staining of the exogenous proliferation marker iododeoxyuridine (IdUrd) and of thymidine kinase 1 (TK-1), the enzyme phosphorylating 18FLT, in primary resection specimen of oral cavity tumors.
Figure 6.1  
$^{18}$FLT-PET-CT scans of patients with primary squamous cell carcinomas of the oral cavity with different SUV$_{\text{max}}$. Left panel: contrast-enhanced CT, middle panel: $^{18}$FLT-PET scan, right panel: fusion of both images; window-level-setting were kept identical. A: pT2N1 floor of mouth, SUV$_{\text{max}}$ 2.2 (patient 12); B: pT4N0 hard palate, SUV$_{\text{max}}$ 6.8 (patient 9); C: pT2N2b floor of mouth, SUV$_{\text{max}}$ 14.7 (patient 7). Note $^{18}$FLT-uptake in bone marrow of the cervical spine.
Figure 6.2 Immunohistochemical staining for TK-1 (left panels; brown), IdUrd (right panels; brown) and nuclei (blue) in adjacent sections of squamous cell carcinomas of the oral cavity. A: absent staining for TK-1 and intense staining for IdUrd in tumor and adjacent inflammatory cells; B: weak staining for TK-1 and intense staining for IdUrd in tumor cells; C: intense staining for TK-1 and IdUrd in tumor cells; D: positive staining for both markers (TK-1 weak, IdUrd strong) in the invasive front of a squamous cell carcinoma.
6.2 Material and Methods

Patients. From July 2005 until March 2008, 17 patients with newly diagnosed clinical stage II-IV primary squamous cell carcinoma of the oral cavity eligible for surgical tumor resection were included in this study after giving written informed consent. The Institutional Review Board of the Radboud University Nijmegen Medical Centre approved this study.

\(^{18}\)FLT synthesis. \(^{18}\)FLT was obtained from the Department of Nuclear Medicine and PET Research, VU University Medical Centre, Amsterdam, the Netherlands. Synthesis was performed according to the method of Machulla et al.\(^{16}\). \(^{18}\)FLT was produced by \(^{18}\)F-fluorination of the 4,4'-dimethoxytrityl protected anhydrothymidine, followed by a deprotection step. After purification by reversed phase high performance liquid chromatography, the product was made isotonic and passed through a 0.22 µm filter. \(^{18}\)FLT was routinely produced with a non-decay corrected radiochemical yield of 5-10%, a radiochemical purity of >97% and a specific activity higher than 10,000 GBq/mmol.

PET/CT acquisition. Prior to the surgical tumor resection, integrated PET and CT images were acquired on either a hybrid PET-CT scanner, or by means of software fusion of dedicated stand-alone PET and CT images. All scans were performed with the patient in supine position and fixated in a rigid customized mask covering the head/neck area in order to increase position accuracy and to reduce movement artifacts during PET-CT scanning.

Hybrid PET/CT images were acquired using a Siemens Biograph Duo scanner (Siemens/CTI, Knoxville, TN). Emission images of the head and neck area were recorded 60 minutes after intravenous injection of approximately 250 MBq \(^{18}\)FLT, with 7 minutes per bed position in 3D mode. PET images were reconstructed using the OSEM iterative algorithm with parameters optimized for the head and neck area (i.e., 4 iterations, 16 subsets and 5 mm 3D Gaussian filter\(^{17}\), with correction for photon attenuation. In addition, CT images were acquired for anatomical correlation and attenuation correction purposes using 80 mAs, 130 kV, slice width 3 mm, and intravenous contrast agent in the venous phase (Optiray, Mallinckrodt Inc., Hazelwood, MO).

Dedicated PET images were acquired using a Siemens ECAT Exact scanner (Siemens/CTI, Knoxville, TN). Emission and transmission images of the head and neck area were recorded 60 minutes after intravenous injection of approximately 250 MBq \(^{18}\)FLT, with 5 minutes per bed position in 3D mode for emission and 3 minutes per bed position in 2D mode for transmission.
PET image reconstruction parameters were identical to the hybrid PET/CT. Dedicated CT images were acquired using a Philips AcQsim CT scanner (Philips, Cleveland, OH), applying the same acquisition parameters as for the hybrid PET/CT. PET and CT image sets were anatomically co-registered using Iterative Closest Point-based optimization of surface maps derived from PET transmission and CT images, with an average registration accuracy of 3 mm.

**PET tracer uptake analysis and image segmentation.** After reconstruction and co-registration, SUV-PET images were created with in-house developed software correcting for injected dose, tracer decay, and patient body weight. Subsequently, these SUV-PET images were resliced using the CT image as reference. SUV-PET and CT images were imported into Pinnacle3 (version 8.0d; Philips Radiation Oncology System, Madison, WI), the radiotherapy planning system routinely used at our department.

The gross tumor volume (GTV) was first delineated on CT (GTV_{CT}). Self-written scripts in Pinnacle3 aided with the segmentation of the primary tumor and with the calculation of the mean and maximum standardized uptake value (SUV_{mean} and SUV_{max}) from the PET data. Two previously described methods for segmentation of PET images were used. First, the 50% isocontour (GTV_{50%}) was based on a fixed percentage of the maximum signal intensity in the gross tumor volume. Second, an adaptive threshold delineation based on the signal-to-background ratio was performed (GTV_{SBR})

For this means, the SUV_{max} was defined as mean activity of the hottest voxel in the tumor and its eight surrounding voxels in a transversal slice. Mean background activity was acquired in a manually defined region of interest in the left neck musculature (approximately 10 cm^3) at sufficient distance from the primary tumor, metastatic cervical lymph nodes and vertebrae. Within the segmented volumes, the mean and maximum standardized uptake values were calculated.

**Administration of IdUrd and immunohistochemical staining of IdUrd and TK-1.**

Twenty minutes before the start of surgery, IdUrd (Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland), 200 mg diluted in 100 mL NaCl 0.9%, was administered intravenously as bolus injection.

The paraffin blocks containing the resection material from the 17 primary oral cavity tumors were collected from the Pathology Department. In order to obtain a representative sample of the tumor, we aimed at collecting three blocks per patient, one from the tumor center and two localized more peripherally.

From these blocks, 5 μm consecutive sections were stained for IdUrd and TK-1. Between all steps of this procedure, primary tumor sections were rinsed with 0.1 M phosphate-buffered saline (PBS; Klinipath Duiven, The Netherlands), pH 7.4. The staining procedure was performed at room temperature unless stated differently. The sections were deparaffinized and re-hydrated in histosafe and graded alcohols (100%...
96% - 70%). For antigen retrieval, slides were heated (90°C) in 10 mM citrate buffer pH 6.0 for 30 min (DAKO, Glostrup, Denmark).

For IdUrd staining, sections were incubated with 2 N HCl for 30 min, with 0.1 M Borax for 15 min, and with normal donkey serum 5% diluted in primary antibody diluent (PAD; GeneTex Inc., San Antonio, TX) at 37 °C for 30 min. Then, sections were incubated with mouse-anti-IdUrd (Caltag Laboratories, Burlingame, CA), diluted 1:3000, for 60 min and peroxidase was blocked with 3% H2O2 in methanol for 10 min.

For TK-1 staining, endogenous peroxidase was blocked with 3% H2O2 in methanol for 10 min, followed by pre-incubation with PAD and incubation with 5% normal donkey serum diluted in PAD for 30 min. Finally, sections were incubated overnight with mouse anti-TK-1 (AbD Serotec, Raleigh, NC), diluted 1:800 in PAD at 4°C.

Next, all sections were incubated with donkey-anti-mouse-biotin (Jackson Immuno Research Laboratories, West Grove, PA), diluted 1:400 (IdUrd) or 1:200 (TK-1) in PBS, for 60 min and subsequently with ABC-reagent (Vector Laboratories, Burlingame, CA) for 30 min. Then, sections were rinsed with deionized water before incubation with diaminobenzidine (DAB; Zymed Laboratories) for 15 min. Finally, after rinsing with tap water and staining with hematoxylin (Klinipath) for 30 sec, sections were dehydrated and captured in mounting medium (Klinipath).

**Immunohistochemical image acquisition and image processing**

**IdUrd labeling index and optical density.** After immunohistochemical staining, the entire tumor sections were scanned at 200 x magnification using a digital image processing system consisting of a color CCD camera (Retiga SRVmm 1392 x 1040 pixels) and a RGB filter (Slider Module; QImaging, Burnaby, BC, Canada) attached to a motorized bright field microscope (DM6000 Leica, Wetzlar, Germany). Whole tumor sections were scanned using a Macintosh computer running IPLab (Scanalytics Inc., Fairfax, VA), which controlled this motorized system and generated 24-bit color composite images. For every scan session, separate background images were recorded.

To extract and separate the color information from the DAB and hematoxylin signals, the RGB linear unmixing module in the TRI2-software was applied using the “Absorption” mode (Randall Division and Gray Cancer Institute, London, UK). For this procedure reference files from single-stained control sections were used containing color information for the blue (hematoxylin; all nuclei) and brown (IdUrd in proliferating nuclei) signal. Additionally, each RGB color image was corrected for the microscope illumination using the background image and was subsequently normalized. Next, threshold values for the DAB and hematoxylin signal were manually set. For the image analysis, a tumor mask was delineated including tumor, intratumoral stroma as well as areas of necrosis but excluding surrounding normal tissues. Within
Validation of \(^{18}\text{FLT}\) in oral cavity tumors

this mask, the labeling index for IdUrd was calculated by dividing the DAB-positive area (nuclei positive for IdUrd) by the hematoxylin area (all nuclei).

In order to adjust for different sizes of immunohistochemically stained nuclei and for variation in the staining intensity for IdUrd, mean optical densities were calculated for the entire tumor area using a Macintosh computer running IPLab.

**Analysis of immunohistochemical staining for TK-1.** Due to a relatively weak immunohistochemical staining of TK-1 in most of the tumors, automated quantitative analyses were not possible. Therefore, two investigators, who were blinded for the results obtained by PET and IdUrd immunohistochemistry, performed a visual and semi-quantitative analysis. The entire tumor sections were assessed using 200 × magnification, and the intensity of the staining (no, weak, strong), the percentage of positive cells and their localization were estimated. The combination of staining intensity with percentage of positive cells resulted in four categories: absent TK-1 staining, weak and sparse (≤5%), weak and abundant (>5-20%), and strong and abundant (>5-20%) staining. There were no tumors with strong and sparse TK-1 staining. Agreement on all tumor sections was achieved before further analysis. TK-1 staining categories were subsequently correlated to the SUV\(_{\text{max}}\) of \(^{18}\text{FLT}\)-PET, the IdUrd labeling indices and the IdUrd optical densities.

**Statistical analysis.** Statistical analyses were performed using GraphPad Prism Version 4.0c for Macintosh (La Jolla, CA). Gaussian distribution of the values was analyzed using the Kolmogorov-Smirnov test. Comparison of the GTV\(_{\text{CT}}\) and GTV\(_{\text{sbr}}\) was performed using a two-tailed Mann Whitney test. Correlation of SUV for PET with IdUrd labeling indices was performed with Pearson correlation. Linear regression was performed for the SUV and IdUrd optical densities under the assumption that an optical density of zero resulted in a standardized uptake value of zero for \(^{18}\text{FLT}\)-PET. The results of the semi-quantitative analysis for TK-1 versus the SUV\(_{\text{max}}\), IdUrd labeling indices and optical densities were analyzed using one-way ANOVA. A p-value ≤ 0.05 was regarded statistically significant.

### 6.3 Results

#### 6.3.1 Patients and tumor characteristics

Thirteen male and four female patients with an average age of 57 years (range 43 to 75 years) participated in this study. Patient and tumor characteristics are given in Table 6.1. Most tumors were clinically and pathologically classified as T2. Patients 1-3 were scanned on the dedicated CT- and PET-machines on the same day, the other patients underwent the \(^{18}\text{FLT}\)-PET-CT scan on the hybrid scanner. The median interval between PET-CT scan and surgery was five days (mean interval: eight days, range 2-36 days).
Chapter 6

The longest interval of 36 days was caused by an intercurrent infection, which delayed the surgery.

Table 6.1  Patient characteristics, clinical and pathological stages, and therapeutic procedures

<table>
<thead>
<tr>
<th>Patient</th>
<th>Site</th>
<th>Gender</th>
<th>Age</th>
<th>Surgical Procedure</th>
<th>Clinical stage</th>
<th>Pathological stage</th>
<th>Tumor grade</th>
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<tbody>
<tr>
<td>1</td>
<td>Lower alveolar ridge</td>
<td>m</td>
<td>46</td>
<td>TE + MRND right</td>
<td>T4N3bM0</td>
<td>T4N0M0</td>
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<tr>
<td>2</td>
<td>Floor of mouth</td>
<td>f</td>
<td>53</td>
<td>TE + bilateral SND</td>
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<td>T2N0M0</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
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<td>m</td>
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<td>TE + SND right</td>
<td>T3N0M0</td>
<td>T3N1M0</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
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<tr>
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<td>T2N0M0</td>
<td>T2N0M0</td>
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<td>m</td>
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<td>T4N0M0</td>
<td>T4N0M0</td>
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<tr>
<td>10</td>
<td>Tongue / floor of mouth</td>
<td>m</td>
<td>62</td>
<td>TE + SND right + MRND right</td>
<td>T2N1M0</td>
<td>T2N2bM0</td>
<td>3</td>
</tr>
<tr>
<td>11</td>
<td>Tongue</td>
<td>f</td>
<td>45</td>
<td>TE + bilateral MRND</td>
<td>T4N2cM0</td>
<td>T4N2cM0</td>
<td>3</td>
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<tr>
<td>12</td>
<td>Floor of mouth</td>
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<td>53</td>
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<tr>
<td>13</td>
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<tr>
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<td>TE + MRND left</td>
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<td>T2N2bM0</td>
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<td>TE + SND left</td>
<td>T2N0M0</td>
<td>T1N1M0</td>
<td>2</td>
</tr>
</tbody>
</table>

f = female, m = male; TE = tumor excision; MRND = modified radical neck dissection; SND = supraomohyoid neck dissection (level I-III).

6.3.2 18FLT uptake in primary tumor

18FLT-PET data were available for all 17 patients, and all primary tumors were detected by 18FLT-PET, as opposed to only 12 by contrast enhanced CT (Fig. 6.1). Results obtained for the signal-to-background segmentation method (SBR) were almost identical to the 50% method (data on file). Therefore, only the SBR data are further discussed and shown. The tumor volume delineated on CT [mean GTV_{CT} (± standard deviation; SD): 13.6 cm³ (± 8.2 cm³)] was significantly larger than the mean GTV_{SBR} [7.2 cm³ (± 5.6 cm³); p = 0.03]. The mean 18FLT-SUV_{max} (± SD) in the primary tumors was 5.9 (± 3.2) and the mean SUV_{mean} within the GTV_{SBR} was 4.0 (± 2.2).
6.3.3 IdUrd labeling index and optical density and correlations with \(^{18}\text{FLT-PET}\)

No adverse events occurred during the intravenous administration of IdUrd or \(^{18}\text{FLT}\). From all 17 patients, paraffin blocks containing primary tumor tissue were available. We aimed at collecting three blocks per patient, one from the tumor center and two more peripherally. However, only one block was available for six patients, two blocks were retrieved for seven patients, and three tumor blocks were available for the remaining four patients, since the tumor tissue was only present in one or two blocks in most cases. In all tumor sections, tumor cells stained positively for IdUrd, and in two cases, IdUrd staining was also present in peritumoral inflammatory tissue (Fig. 6.2A). There was a large variability in IdUrd labeling indices with a mean value of 0.09 and a standard deviation of ± 0.14 (range 0.01-0.29). Furthermore, there was a large range in optical densities for IdUrd, with a mean of 28.2 (± 12.7; range 12.6-37.8).

There was no significant correlation between the mean IdUrd labeling index and the \(\text{SUV}_{\text{max}}\) or \(\text{SUV}_{\text{mean}}\) of \(^{18}\text{FLT-PET}\) (Fig. 6.3A; data for \(\text{SUV}_{\text{mean}}\) not shown). In contrast to this, the signal intensities, i.e., optical densities for immunohistochemical IdUrd staining versus \(^{18}\text{FLT-PET SUV}_{\text{max}}\) and \(\text{SUV}_{\text{mean}}\) demonstrated a significant albeit weak correlation \((p < 0.0001; \text{Fig. 6.3B}; \text{data for } \text{SUV}_{\text{mean}} \text{ not shown})\).

6.3.4 TK-1 staining and correlations with IdUrd and \(^{18}\text{FLT-PET}\)

In thirteen of the seventeen squamous cell carcinomas of the oral cavity, TK-1 staining was found in the cytoplasm of tumor cells, with an intensity ranging from weak to intense (Fig. 6.2A-C). In one tumor, TK-1 staining was predominantly localized in the invasive tumor front (Fig. 6.2D). Overall, staining for TK-1 was substantially weaker compared to IdUrd and less tumor cells stained positive. Mostly, TK-1 and IdUrd co-localized in the same tumor areas, but correlation at the cellular level could not be assessed, because the stainings were performed on consecutive sections.

After semi-quantitative analysis of TK-1 staining, four categories were identified based on staining intensity and percentage of positive cells. These categories were correlated to the \(\text{SUV}_{\text{max}}\) of \(^{18}\text{FLT-PET}\) (Fig. 6.4A), the IdUrd labeling indices (Fig. 6.4B) and the IdUrd optical densities (Fig. 6.4C). However, no statistically significant correlations between these parameters were found.
6.4 Discussion

$^{18}$FLT is a potential PET-tracer for non-invasive tumor characterization and treatment response monitoring related to tumor cell proliferation. In primary squamous cell carcinomas of the oral cavity, we validated $^{18}$FLT PET against immunohistochemical staining of the exogenous proliferation marker IdUrd and expression of the TK-1 enzyme that is involved in DNA synthesis.

In oral cavity tumors, we found a relatively high mean SUV$_{\text{max}}$ of 5.9 (range 2.2-15.2). The sensitivity for tumor detection was high (100%) as compared to contrast-enhanced CT (76%) that was hampered by the relatively small tumor size and dental artifacts. Lower SUV$_{\text{max}}$ (mean maximum SUV 1.6; range 1.0-5.7) were observed in laryngeal tumors with a lower sensitivity (88%), possibly due to the generally smaller volumes of larynx carcinomas. Two other publications involving head and neck cancers of various sites and non-small cell lung cancer both reported a mean SUV$_{\text{max}}$ of 4.8.

The gross tumor volumes delineated after segmentation of the PET signal (GTV$_{\text{SSR}}$) were significantly smaller than the GTVs manually delineated on contrast enhanced CT scans.
A similar finding has previously been reported for laryngeal tumors using \(^{18}\)FDG-PET. Daisne et al. correlated true tumor volumes measured in laryngeal resection specimen with GTVs delineated on anatomical (CT, MRI) and functional imaging modalities (PET). The GTVs using PET were the smallest compared to CT and MRI, and the most accurate compared to histology.

However, the potential application of \(^{18}\)FLT-PET in oncology may not be so much in improving the sensitivity of tumor detection or its anatomical extensions, but more in its ability to characterize the biological behavior of a tumor. Therefore, we compared quantitative analysis of \(^{18}\)FLT-uptake with standard methods for quantification of proliferation by immunohistochemical markers. IdUrd is an S-phase specific and robust marker of proliferation. It was hypothesized that IdUrd binding should correlate with \(^{18}\)FLT-uptake, as the activity of TK-1, the key enzyme in \(^{18}\)FLT phosphorylation, is also mainly upregulated during the S-phase. Somewhat unexpectedly, we did not find a correlation between the IdUrd labeling indices and the \(^{18}\)FLT SUV\(_{\text{max}}\) or SUV\(_{\text{mean}}\). In contrast to our findings, several studies on different tumor types found significant positive correlations between standardized uptake values for \(^{18}\)FLT-PET and proliferation assessed by Ki-67 immunohistochemistry (Table 6.2). Three other studies in thoracic, breast and gastric tumors did not demonstrate such a correlation.

There are a number of factors that potentially play a role in the lack of correlation between IdUrd labeling indices and \(^{18}\)FLT-PET standardized uptake values in the current study. (1) All other validation studies were based on the endogenous proliferation marker Ki-67 that is upregulated during various phases of the cell cycle (G\(_1\), S, G\(_2\), M), and for which labeling indices of up to 30% have been reported. The exogenous proliferation marker IdUrd is only incorporated during the S-phase of the cell cycle, and therefore theoretically should correlate better with \(^{18}\)FLT-uptake. This was not confirmed in the current study. Subsequently, we also stained the primary tumor sections for Ki-67 and calculated the labeling index by the same automated analysis methodology as described for IdUrd. However, analysis of Ki-67 revealed similar or slightly worse correlations with \(^{18}\)FLT-uptake compared to IdUrd (data on file).

(2) For most patients, only one or two paraffin-embedded blocks containing tumor tissue were available and from each block one single section was analyzed. Thus, only a relatively small tumor volume was assessed compared to the entire tumor volume characterized by \(^{18}\)FLT-PET, with the inherent risk of sampling error. However, in contrast to studies by others, we tried to minimize this error by analyzing at least two tumor-containing paraffin blocks in 11 of the 17 patients and by examining the entire tumor sections (Table 6.2).
(3) There was an interval between PET scanning and surgery of several days. However, compared to other studies reported in the literature, the median interval of 5 days was relatively short and we do not expect the tumor’s proliferative activity to change substantially during this period.

(4) The resolution of the imaging modalities profoundly differed, i.e., 2.5 μm for automatic analysis of bright field microscopy of immunohistochemical sections as opposed to 5-8 mm using PET.

(5) Different quantification procedures may influence the results. Some investigators assessed the mean labeling index or mean standardized uptake value, while others calculated the respective maximum values. Furthermore, these values were mostly estimated in the areas of highest proliferative activity or greatest staining intensity as opposed to complete tumor sections in our study. To specifically address this question, we also correlated the IdUrd labeling indices in the areas of highest proliferative activity with $^{18}$FLT SUV, but again, this did not result in significant correlations (data on file). The IdUrd labeling index is a measure of the relative number of tumor cells in S-phase, as opposed to the $^{18}$FLT standardized uptake value, which reflects the signal intensity of the tracer at a certain time point. $^{18}$FLT uptake is determined by the tumor cells’ DNA synthesis rate and might be better represented by the IdUrd staining intensity and less by labeling index. In order to test this, we assessed the IdUrd optical densities as a measure of staining intensity within the previously defined tumor areas. This approach yielded statistically significant albeit weak correlations with SUV$_{\text{max}}$ and SUV$_{\text{mean}}$ for $^{18}$FLT (Fig. 6.3B). However, no significant results were found when comparing Ki-67 optical densities with $^{18}$FLT standardized uptake values (data on file).

Finally, we assessed the expression of TK-1, the key enzyme involved in the exogenous (salvage) pathway of DNA synthesis $^{13}$. We studied the intensity of staining, its localization and the global co-localization with IdUrd. The majority of the tumors stained positive for TK-1, although most of the staining was rather faint and only present in a few tumor cells. In the consecutive tumor sections, the area of cytoplasmatic TK-1 staining co-localized with nuclear staining for IdUrd on a global level, but correlation on a cellular level could not be assessed. There was no correlation between TK-1 staining intensity and number of TK-1 positive cells on the one hand, and the $^{18}$FLT SUV$_{\text{max}}$, the IdUrd labeling indices or the IdUrd optical densities on the other.

TK-1 staining has been used for flow cytometric analyses, but experience with immunohistochemical staining for TK-1 in tumor sections is very limited $^{38}$. A few groups have performed immunohistochemistry on histological tumor sections of breast, non-small cell lung cancer and colorectal cancer patients $^{39-43}$. Statistically significant correlations with Ki-67 and the proliferating cell nuclear antigen (PCNA) were found $^{40-43}$. The TK-1 labeling indices reported in these four studies were high (50-80%) compared to our experience in squamous cell carcinomas of the oral cavity $^{40-43}$. To date,
only one study on head and neck squamous cell carcinoma xenografts has been published. Molthoff et al. assessed the value of \(^{18}\)FLT-PET for treatment response monitoring in xenografted squamous cell carcinomas after fractionated irradiation and immunohistochemically stained tumor sections for TK-1. The authors reported a significant decrease in \(^{18}\)FLT tumor-to-normal-tissue ratios, while TK-1 expression in control and irradiated tumors remained similar.

<table>
<thead>
<tr>
<th>Table 6.2</th>
<th>Published validation studies on (^{18})FLT-PET</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Positive correlation</strong></td>
<td></td>
</tr>
<tr>
<td>Author</td>
<td>Primary tumor site</td>
</tr>
<tr>
<td>Buck 25</td>
<td>NSCLC</td>
</tr>
<tr>
<td>Chen 26</td>
<td>Cerebral glioma</td>
</tr>
<tr>
<td>Cobben 27</td>
<td>Soft tissue sarcoma</td>
</tr>
<tr>
<td>Eckel 28</td>
<td>Hepatocellular carcinoma</td>
</tr>
<tr>
<td>Kenny 29</td>
<td>Breast cancer</td>
</tr>
<tr>
<td>Muzi 30</td>
<td>NSCLC</td>
</tr>
<tr>
<td>Vesselle 31</td>
<td>NSCLC</td>
</tr>
<tr>
<td>Yamamoto 32</td>
<td>NSCLC</td>
</tr>
<tr>
<td>Yap 33</td>
<td>NSCLC and pulmonary lesions</td>
</tr>
<tr>
<td><strong>No correlation</strong></td>
<td></td>
</tr>
<tr>
<td>Dittmann 34</td>
<td>Thoracic tumors (incl. nine NSCLC)</td>
</tr>
<tr>
<td>Kameyama 35</td>
<td>Gastric cancer</td>
</tr>
<tr>
<td>Smyczek-Gargya 36</td>
<td>Breast cancer</td>
</tr>
</tbody>
</table>

\(N^0 = \) number; NSCLC = non-small cell lung cancer.

However, details regarding TK-1 staining intensity, localization and labeling index were not provided. Based on the current results, the value of TK-1 staining as a measure of proliferative activity in oral cavity tumors seems limited. However, further research is
needed (e.g., studies on co-localization with standard proliferation markers at the cellular level) until definite conclusions can be drawn about its significance as a biomarker for tumor cell proliferation.

6.5 Conclusions

Non-invasive $^{18}$FLT-PET imaging assessing the proliferative status of tumors may be a valuable aid for patient selection and treatment response monitoring in head and neck cancer, but it needs to be validated before introduction in the clinic. This validation study demonstrated only a weak correlation between $^{18}$FLT-uptake and IdUrd staining intensity in squamous cell carcinomas of the oral cavity. Immunohistochemical staining of TK-1, the key enzyme in $^{18}$FLT phosphorylation, did neither correlate with $^{18}$FLT-uptake nor with the standard immunohistochemical proliferation markers. This may be due to differences in biomarker characteristics, discrepancies in resolution of the imaging modalities, and differences in quantification methods. The potential role of $^{18}$FLT-PET in clinical oncology remains to be further explored.

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6.6 References

Validation of $^{18}$FLT in oral cavity tumors


Chapter 7

$^{18}$FLT-PET does not discriminate between reactive and metastatic lymph nodes in primary head and neck cancer patients

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Abstract

Introduction. Repopulation of clonogenic tumor cells is inversely correlated with radiation treatment outcome in head and neck squamous cell carcinomas. A functional imaging tool to assess the proliferative activity of tumors could improve patient selection for treatment modifications and could be used for early treatment response evaluation. The PET tracer 3'-deoxy-3'-18Fluorothymidine (18FLT) can image tumor cell proliferation prior to and during radiotherapy, and it may provide biological tumor information useful in radiotherapy planning. In the present study, the value of 18FLT-PET in determining the lymph node status in squamous cell carcinoma of the head and neck was assessed, with pathology as the gold standard.

Materials and Methods. Ten patients with newly diagnosed stage II-IV squamous cell carcinoma of the head and neck underwent 18FLT-PET prior to surgical tumor resection with lymph node dissection. Emission 18FLT-PET and CT images of the head and neck were recorded and fused, and standardized uptake values (SUV) were calculated. From all 18 18FLT-PET positive lymph node levels and from eight 18FLT-PET negative controls, paraffin embedded lymph node sections were stained and analyzed for the endogenous proliferation marker Ki-67 and for the pre-operatively administered proliferation marker iododeoxyuridine. Sensitivity, specificity, positive predictive value and negative predictive value were calculated for 18FLT-PET.

Results. Primary tumor sites were oral cavity (7), larynx (2) and maxillary sinus (1). Nine of the ten patients examined had 18FLT-PET positive lymph nodes (SUV$_{\text{mean}}$: median 1.2, range 0.8-2.9), but only three of these patients had histologically proven metastases. All metastatic lymph nodes showed Ki-67 and iododeoxyuridine staining in tumor cells. In the remaining seven patients, there was abundant Ki-67 and iododeoxyuridine staining of B-lymphocytes in germinal centers in PET positive lymph nodes explaining the high rate of false positive findings. Sensitivity, specificity, positive and negative predictive values of 18FLT-PET were 100%, 16.7%, 37.5% and 100%, respectively.

Conclusions. In head and neck cancer patients, 18FLT-PET scan showed uptake in metastatic as well as in non-metastatic reactive lymph nodes, the latter due to reactive B-lymphocyte proliferation. Because of the low specificity, 18FLT-PET is not suitable for assessment of pretreatment lymph node status. This observation may also negatively influence the utility of 18FLT-PET for early treatment response evaluation of small metastatic nodes.
7.1 Introduction

Lymph node involvement in squamous cell carcinoma of the head and neck is a poor prognostic indicator, reducing cure rate by almost 50%. Standard diagnostic work-up for assessing cervical lymph node status is performed by computed tomography (CT) or magnetic resonance imaging (MRI). The sensitivity (50%-80%) and specificity (70%-90%) of CT and MRI are comparable. For marginally enlarged lymph nodes, examination by ultrasound imaging (US) with fine needle aspiration cytology, is superior to CT and MRI if performed by an experienced radiologist (sensitivity and specificity up to 76% and 100%, respectively). More recently, a number of studies have been performed to assess the value of positron emission tomography with 18F-fluorodeoxyglucose (18FDG-PET) for cervical lymph node staging. The results of these studies indicate that the performance of 18FDG-PET is not clearly superior to US, CT or MRI. Therefore, 18FDG-PET is generally not considered as part of the standard work-up for head and neck cancer patients for this indication.

An additional biologic factor of prognostic relevance is tumor cell proliferation. Head and neck squamous cell carcinomas show accelerated repopulation of clonogenic tumor cells during the course of radiation therapy and this is related to poor treatment outcome. Several treatment modifications have been developed to counteract this phenomenon, such as accelerated radiotherapy and inhibition of the epidermal growth factor receptor (EGFR), but at the cost of increased toxicity for the patient. Hence, careful patient selection for these treatment strategies is required ensuring maximal patient benefit, while preventing undue toxicity and costs. A diagnostic tool to identify lymph node metastases with high accuracy that can also provide information on the proliferative activity of the tumor could be of great value for treatment selection and radiotherapy planning.

Shields et al. introduced the novel PET-tracer 3′-deoxy-3′-18F-fluorothymidine (18FLT) that is monophosphorylated by the cytosolic enzyme thymidine kinase 1 (TK-1) and trapped intracellularly. TK-1 activity is increased during DNA synthesis and 18FLT trapping is related to TK-1 activity and thus to proliferation. It is of importance to note that 18FLT is not incorporated into the DNA and that this TK-1 mediated pathway could theoretically be upregulated although DNA synthesis is inhibited. A number of studies evaluated the usefulness of 18FLT in assessing tumor cell proliferation in the primary tumor, most of them including a comparison with 18FDG-PET. Several studies validated 18FLT tracer uptake with the proliferation marker Ki-67 in primary tumor resection material or biopsies. A single study on laryngeal carcinoma by Cobben et al. compared 18FLT with 18FDG-PET for imaging of the primary tumor without histological verification. Only three studies, two on breast carcinoma and one on
thoracic tumors, have validated $^{18}$FLT-PET versus histopathology for the detection of metastatic lymph nodes $^{20,30,31}$.

Different markers have been used for histological assessment of proliferation. These include endogenous markers such as Ki-67, proliferating cell nuclear antigen (PCNA) and members of the cyclin group, or intravenous administration of the thymidine analogues bromodeoxyuridine (BrdUrd) and iododeoxyuridine (IdUrd) $^{36-38}$. The latter have a short half-life and are rapidly incorporated in the DNA of S-phase cells $^{39}$. For immunohistochemical validation of $^{18}$FLT these thymidine analogues seem most suitable because TK-1 activity is increased mainly during DNA synthesis.

Thus far, validation of the PET-tracer $^{18}$FLT has mainly focused on primary tumor sites and only recently this was expanded to determining lymph node status. Characterization of both the primary tumor and the lymph nodes is compulsory for selection of the treatment strategy in patients with squamous cell carcinomas of the head and neck. The aim of this study was to determine the value of $^{18}$FLT-PET for assessment of the cervical lymph node status and proliferative activity with histological evaluation as the gold standard.

**Figure 7.1** $^{18}$FLT-PET-CT images of patient number 9 (pT2N0M0 oral cavity carcinoma). The upper panels show the PET-images, the middle panels the CT-images and the lower panels the fusion of both image modalities. Cervical lymph nodes with increased $^{18}$FLT uptake are found bilaterally in level II (A: indicated by white arrows) and in levels III and IV (B: white arrows in the coronal images). All lymph nodes detected with $^{18}$FLT in this example were false-positive for metastasis, due to uptake in proliferating B-lymphocytes in reactive germinal centers.
Figure 7.2  Ki-67 and IdUrd staining in: A: germinal center harboring proliferating B-lymphocytes and remaining lymphoid tissue; B: remaining lymphoid tissue with proliferating lymphoid cells; C: metastasis of a squamous cell carcinoma of the maxillary sinus; D: micrometastasis with keratinization (indicated by purple arrow), fragment of a germinal center (white arrow) and surrounding lymphoid tissue.
7.2 Material and Methods

Patients. Ten patients with newly diagnosed stage II-IV primary squamous cell carcinoma of the head and neck awaiting surgical tumor and lymph node resection were included in this study after giving written informed consent. The study was approved by the Institutional Review Board of the Radboud University Nijmegen Medical Centre, the Netherlands.

\(^{18}\)FLT synthesis. \(^{18}\)FLT was obtained commercially from the Cyclotron B.V., VU Medical Centre, Amsterdam, The Netherlands. Synthesis was performed according to the method of Machulla et al. \(^{40}\). In brief, \(^{18}\)FLT was produced by \(^{18}\)fluorination of the 4,4'-dimethoxytrityl protected anhydrothymidine, followed by a deprotection step. After purification by reversed phase high performance liquid chromatography, the product was made isotonic and passed through a 0.22 \(\mu\)m filter. \(^{18}\)FLT was produced with a non-decay corrected radiochemical yield of 5 - 10\%, a radiochemical purity of >95\% and a specific activity of >10 TBq/mmol.

PET/CT acquisition. Prior to surgical tumor resection, integrated PET and CT images were acquired with either a hybrid PET/CT (patient 7, 9, 10), or with software fusion of dedicated PET and CT images (all remaining patients). All scans were performed with the patient positioned in a rigid customized mask covering the head/neck area in order to increase position accuracy and to reduce movement artifacts during PET-scanning.

Hybrid PET/CT images were acquired using a Siemens Biograph Duo scanner (Siemens/CTI, Knoxville, TN). Emission images of the head and neck area were recorded 60 minutes after intravenous injection of 250 MBq \(^{18}\)FLT, with 4 minutes per bed position in 3D mode. PET images were reconstructed using the OSEM iterative algorithm with parameters optimized for the head and neck area (i.e., 4 iterations, 16 subsets and 5 mm 3D Gaussian filter), with correction for photon attenuation. In addition, CT images were acquired with 80 mAs, 130 kV, and slice width 3 mm, with i.v. contrast in the venous phase, for anatomical correlation and attenuation correction purposes.

Dedicated PET images were acquired using a Siemens ECAT Exact scanner (Siemens/CTI, Knoxville, TN). Emission and transmission images of the head and neck area were recorded 60 minutes after intravenous injection of 250 MBq FLT, with 5 minutes per bed position in 3D mode for emission and 3 minutes per bed position in 2D mode for transmission. PET image reconstruction was identical to images from hybrid PET/CT. Dedicated CT images were acquired using a Philips AcQsim CT scanner (Philips, Cleveland, OH), with the same acquisition parameters as CT images from hybrid PET/CT. PET and CT image sets were anatomically co-registered using Iterative Closest Point-based optimization of surface maps derived from PET transmission and CT images, with an average registration accuracy of 3 mm \(^{41}\).
**PET analysis.** Before analyzing the histological sections, the combined PET/CT image sets were reviewed in consensus by two experienced observers blinded for all patient data. Images were scored for presence or absence of $^{18}$FLT-PET uptake. Lymph node levels were determined as described by Gregoire et al. $^{42}$ Maximum and mean standardized uptake values ($SUV_{\text{max}}$ and $SUV_{\text{mean}}$) were calculated for visible lymph nodes. $SUV_{\text{mean}}$ was calculated after constructing a region of interest at the 50% isocontour of the $SUV_{\text{max}}$.

**Surgery.** Twenty minutes before the start of surgery, IdUrd (Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland), 200 mg diluted in 100 mL NaCl 0.9%, was administered intravenously as bolus injection. After resection, the neck dissection specimens were presented on a uniform left- or right-sided plate resembling the neck levels I-VI.

**Immunohistochemical staining of IdUrd and Ki-67.** From the ten patients, a total of 236 lymph nodes without metastases and 14 lymph nodes with metastatic involvement localized in 44 lymph node levels were removed. From these, paraffin blocks containing lymph nodes from 26 different lymph node levels were collected for this study. These included 18 $^{18}$FLT-PET positive lymph node levels and 8 randomly chosen levels that were $^{18}$FLT-PET negative.

From these blocks, five $\mu$m sections were cut and consecutive sections were stained for Ki-67 and IdUrd. Between all consecutive steps of the staining procedure, lymph node sections were rinsed with 0.1 M phosphate-buffered saline (Klinipath, Duiven, The Netherlands), pH 7.4. The staining procedures were performed at room temperature unless stated differently. The sections were deparaffinized and re-hydrated in histosafe and graded alcohols. For antigen retrieval, slides were heated (90 °C) in 10 mM citrate buffer pH 6.0 for 30 min. For the Ki-67 staining, sections were incubated with normal donkey serum 5% diluted in primary antibody diluent (PAD, Abcam, Cambridge, UK) at 37 °C for 30 min. Overnight, sections were incubated with mouse-anti-human-Ki67 (Zymed Laboratories, San Francisco, CA), undiluted, at 4 °C.

For the IdUrd staining, sections were incubated with 2 N HCl for 30 min followed by incubation, with 0.1 M Borax for 15 min and with normal donkey serum 5% diluted in PAD at 37 °C for 30 min. Then, sections were incubated with mouse-anti-IdUrd (Caltag Laboratories, Burlingame, CA), diluted 1:3000, for 60 min. In both staining procedures, peroxidase was blocked with 3% $H_2O_2$ in methanol for 10 min. Next, all sections were incubated with donkey-anti-mouse-biotin (Jackson Immuno Research Laboratories, West Grove, PA), diluted 1:400, for 60 min and with ABC-reagent (Vector Laboratories, Burlingame, CA) for 30 min. Then, sections were rinsed with deionized water before incubation with diaminobenzidine (Zymed Laboratories) for 15 min. Finally, after rinsing with tap water and staining with hematoxylin (Klinipath) for 30 sec, sections were dehydrated and captured in mounting medium (Klinipath).
Pathology evaluation and assessment of proliferation. The removed lymph nodes were routinely stained for hematoxylin and eosin (H&E) and assessed for metastatic involvement by a pathologist. Next, the clinical investigators and an experienced pathologist (PS) reviewed all lymph node sections stained for Ki-67 and IdUrd. Based on 18FLT-PET images and pathologic findings, lymph nodes were assigned to one of three groups: true positive (18FLT positive lymph node with histologically proven metastasis), true negative (18FLT negative lymph node without metastasis) and false positive (18FLT positive lymph node without metastasis) or false negative (18FLT negative lymph node with histologically proven metastasis).

Three histologically distinct areas were distinguished in the lymph node sections: germinal centers, metastatic tumor (if present) and remaining lymphoid tissue (Fig. 7.2). In these areas, Ki-67 and IdUrd positive and negative nuclei were counted using a grid with 25 fields placed in the eyepiece at 100 x magnification (three randomly selected fields were analyzed in germinal centers, three fields in metastatic tissue and one field in remaining lymphoid tissue). The Ki-67 and IdUrd labeling index (LI) was determined as the number of positively stained nuclei relative to the total number of nuclei in a certain area.

In all lymph node sections, the total lymph node area and the (relative) area occupied by germinal centers and, if present, metastatic tumor were calculated. This was done by scanning the entire section under bright field microscopy and reconstructing a composite image of the complete lymph node using image analysis software (IPLab, Scanalytics Inc., Fairfax, VA). Masks were drawn on these scans indicating the total lymph node area, the germinal centers and metastatic tumor deposits. Next, using the image analysis software, the absolute and relative areas occupied by germinal centers and metastatic deposits were calculated.

SUV\text{mean} versus Ki-67 and IdUrd staining. As a measure of total proliferative activity in the germinal centers of a lymph node section, the product of the Ki-67 or IdUrd LI and the absolute area occupied by the germinal centers was calculated. These parameters were called: Ki-67\text{germinal center} and IdUrd\text{germinal center}. Similarly, as a measure of total proliferative activity in the entire lymph node, the sum of the products of LI and absolute area of germinal centers, remaining lymphoid tissue and, if present, metastatic deposits was calculated: Ki-67\text{lymph node} and IdUrd\text{lymph node}. These parameters were compared between true positive, false positive and true negative lymph nodes and were correlated with SUV\text{mean}.

Statistical analysis. The ANOVA-test was used to assess differences in absolute area and Ki-67 and IdUrd LI between true negative, false positive and true positive lymph nodes. The t-test was applied for comparison of number and absolute area of germinal centers and for comparison of Ki-67\text{germinal center} and IdUrd\text{germinal center} between true
negative and false positive lymph nodes. Correlations between Ki-67 lymph node and IdUrd lymph node and SUV mean were calculated using linear regression. All statistical analyses were calculated using GraphPad Prism for Macintosh (version 4.0a; La Jolla, CA). A p-value ≤ 0.05 was regarded statistically significant.

### 7.3 Results

#### 7.3.1 Patients and treatment

Patient characteristics are summarized in Table 7.1. Four men and six women with a mean age of 59 years (range 43-80 years) were included. Primary tumor sites were oral cavity (7), larynx (2) and maxillary sinus (1). Preoperative staging of the neck was performed with ultrasound imaging (8 patients) - with fine needle aspiration cytology in 6 patients and without in two - with CT (3 patients) or MRI (4 patients). All patients underwent surgical tumor resection combined with assessment of cervical lymph node involvement according to Dutch National Guidelines: Six patients with clinically N0 oral cavity carcinoma underwent selective neck dissection of level I-III. A (modified) radical neck dissection was done in three patients with pre-operatively proven cervical lymph node involvement. In one patient with a glottic laryngeal carcinoma without suspected lymph node involvement only sampling of level II and III nodes was performed. Neck surgery was performed unilaterally in seven patients and bilaterally in three patients.

**Table 7.1** Patient characteristics, diagnostic and therapeutic procedures and histopathology of lymph nodes

<table>
<thead>
<tr>
<th>Patient</th>
<th>Site</th>
<th>US and fine needle aspiration cytology (side)</th>
<th>CT</th>
<th>MRI</th>
<th>Clinical stage</th>
<th>Pathological stage</th>
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<tbody>
<tr>
<td>1</td>
<td>Maxillary sinus</td>
<td>+ (left)</td>
<td>n.p.</td>
<td>pos.</td>
<td>T4N1M0</td>
<td>pT4pN1M0</td>
</tr>
<tr>
<td>2</td>
<td>Larynx</td>
<td>n.p.</td>
<td>neg.</td>
<td>n.p.</td>
<td>T3N0M0</td>
<td>pT3pN0M0</td>
</tr>
<tr>
<td>3</td>
<td>Larynx</td>
<td>n.p.</td>
<td>pos.</td>
<td>n.p.</td>
<td>T4N2cM0</td>
<td>pT4pN2cM0</td>
</tr>
<tr>
<td>4</td>
<td>Lower alveolar ridge</td>
<td>- (left)</td>
<td>neg.</td>
<td>n.p.</td>
<td>T4N2bM0</td>
<td>pT4pN0M0</td>
</tr>
<tr>
<td>5</td>
<td>Floor of mouth</td>
<td>n.r.</td>
<td>n.p.</td>
<td>n.p.</td>
<td>T2N0M0</td>
<td>pT2pN0M0</td>
</tr>
<tr>
<td>6</td>
<td>Tongue</td>
<td>- (right)</td>
<td>n.p.</td>
<td>n.p.</td>
<td>T3N0M0</td>
<td>pT2pN0M0</td>
</tr>
<tr>
<td>7</td>
<td>Soft palate</td>
<td>n.p.</td>
<td>n.p.</td>
<td>n.p.</td>
<td>T2N0M0</td>
<td>pT2pN0M0</td>
</tr>
<tr>
<td>8</td>
<td>Tongue</td>
<td>n.p.</td>
<td>n.p.</td>
<td>neg.</td>
<td>T3N0M0</td>
<td>pT3pN1M0</td>
</tr>
<tr>
<td>9</td>
<td>Tongue / Floor of mouth</td>
<td>- (left)</td>
<td>n.p.</td>
<td>neg.</td>
<td>T4N0M0</td>
<td>pT2pN0M0</td>
</tr>
<tr>
<td>10</td>
<td>Tongue / Floor of mouth</td>
<td>- (left)</td>
<td>n.p.</td>
<td>neg.</td>
<td>T2N0M0</td>
<td>pT2pN0M0</td>
</tr>
</tbody>
</table>
Table 7.1  
(continued)

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Pathology LN* (number of pathological LN)</th>
<th>LN positive on PET and available for pathologic assessment (number of LN indicated)</th>
<th>LN positive on PET but not available for pathologic assessment (number of LN indicated)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>left</td>
<td>right</td>
</tr>
<tr>
<td>TE§ + MRND§ left</td>
<td>pos (1)</td>
<td>1</td>
<td>n.a.</td>
</tr>
<tr>
<td>TLE + bilateral NS§</td>
<td>neg</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>TLE + bilateral RND**</td>
<td>pos (7)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>TE + MRND right</td>
<td>n.a.</td>
<td>n.a.</td>
<td>2</td>
</tr>
<tr>
<td>TE + bilateral SNDtt</td>
<td>neg</td>
<td>neg</td>
<td>neg</td>
</tr>
<tr>
<td>TE + SND right</td>
<td>n.a.</td>
<td>n.a.</td>
<td>-</td>
</tr>
<tr>
<td>TE + SND left</td>
<td>neg</td>
<td>n.a.</td>
<td>neg</td>
</tr>
<tr>
<td>TE + SND right</td>
<td>n.a.</td>
<td>pos (1)</td>
<td>-</td>
</tr>
<tr>
<td>TE + SND left</td>
<td>neg</td>
<td>n.a.</td>
<td>3</td>
</tr>
<tr>
<td>TE + SND left</td>
<td>neg</td>
<td>n.a.</td>
<td>2</td>
</tr>
</tbody>
</table>


7.3.2 18FLT-PET

18FLT-PET scans were acquired simultaneously on PET/CT (3) or consecutively on CT and PET (7). The median time interval between 18FLT-PET and surgery was 5 days (average 9.9 days, range 4-37 days). In all but one case, surgery was performed within 4-14 days after the PET-scan. In the case with the longest interval (37 days), the surgical resection of an oral cavity carcinoma was postponed, because the patient underwent laser evaporation of a small laryngeal lesion first. In all but one patient (number 5), increased mean SUV ranging from 0.8-2.9 (median 1.2, standard deviation (± SD) 0.41) were detected, mostly in multiple lymph nodes. A typical 18FLT-PET-CT image is shown in Figure 7.1.

7.3.3 Pathological evaluation

Routine pathology based on H&E staining revealed three patients to have metastatic cervical lymph node disease (numbers 1, 3, 8), whereby clinical examination and anatomical imaging had predicted this only in two patients (numbers 1 and 3). The third patient had a micrometastasis of less than 2 mm in one lymph node (number 8). Although pre-operative staging of the neck revealed multiple ipsilateral enlarged lymph nodes in patient 4, final histology showed no signs of metastatic disease.
In total, paraffin-embedded sections containing 26 lymph node levels were selected for Ki-67 and IdUrd staining and analysis. Eighteen of these levels were positive on $^{18}$FLT-PET, eight levels were negative. As shown in Table 7.2, comparison of $^{18}$FLT-PET results with pathology revealed six true positive, twelve false positive and eight true negative findings.

### Table 7.2  
**Histological LN* assessment: $SUV_{\text{mean}}$, histopathology and mean Ki-67 and IdUrd staining**

<table>
<thead>
<tr>
<th>Patient</th>
<th>LN level</th>
<th>Pathology (metastasis + or -)</th>
<th>$^{18}$FLT-PET</th>
<th>Ki-67 and IdUrd staining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>FLT + SUV&lt;sub&gt;mean&lt;/sub&gt;</td>
<td>FLT - n.a.</td>
</tr>
<tr>
<td>1</td>
<td>R&lt;sup&gt;+&lt;/sup&gt; II</td>
<td>L III</td>
<td>-</td>
<td>(TN&lt;sup&gt;+&lt;/sup&gt;)</td>
</tr>
<tr>
<td></td>
<td>L&lt;sup&gt;-&lt;/sup&gt; II</td>
<td>2.9</td>
<td>+</td>
<td>(TP&lt;sup&gt;+&lt;/sup&gt;)</td>
</tr>
<tr>
<td>2</td>
<td>R II</td>
<td>1.2</td>
<td>-</td>
<td>(FP&lt;sup&gt;-&lt;/sup&gt;)</td>
</tr>
<tr>
<td></td>
<td>R III</td>
<td>0.9</td>
<td>n.a.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R IV</td>
<td>1.2</td>
<td>n.a.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L III</td>
<td>1.1</td>
<td>-</td>
<td>(FP)</td>
</tr>
<tr>
<td>3</td>
<td>R II</td>
<td>1.2</td>
<td>n.a.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R III</td>
<td>1.3</td>
<td>+</td>
<td>(TP)</td>
</tr>
<tr>
<td></td>
<td>R IV</td>
<td>1.1</td>
<td>+</td>
<td>(TP)</td>
</tr>
<tr>
<td></td>
<td>L II</td>
<td>1.7</td>
<td>+</td>
<td>(TP)</td>
</tr>
<tr>
<td></td>
<td>L IV</td>
<td>1.3</td>
<td>+</td>
<td>(TP)</td>
</tr>
<tr>
<td>4</td>
<td>R II</td>
<td>1.4</td>
<td>-</td>
<td>(FP)</td>
</tr>
<tr>
<td></td>
<td>R III</td>
<td>1.3</td>
<td>+</td>
<td>(TP)</td>
</tr>
<tr>
<td></td>
<td>R IV</td>
<td>1.1</td>
<td>+</td>
<td>(TP)</td>
</tr>
<tr>
<td></td>
<td>L II</td>
<td>1.0</td>
<td>n.a.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L IV</td>
<td>0.8</td>
<td>n.a.</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>R I</td>
<td>-</td>
<td>(TN)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>L II</td>
<td>-</td>
<td>(TN)</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>R I</td>
<td>1.4</td>
<td>-</td>
<td>(FP)</td>
</tr>
<tr>
<td></td>
<td>R II</td>
<td>1.3</td>
<td>-</td>
<td>(FP)</td>
</tr>
<tr>
<td></td>
<td>R III</td>
<td>0.9</td>
<td>-</td>
<td>(FP)</td>
</tr>
<tr>
<td>7</td>
<td>R IV</td>
<td>1.5</td>
<td>n.a.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L I</td>
<td>-</td>
<td>(TN)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>L III</td>
<td>-</td>
<td>(TN)</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>R II</td>
<td>1.0</td>
<td>+</td>
<td>(TP)</td>
</tr>
<tr>
<td></td>
<td>R III</td>
<td>0.8</td>
<td>n.a.</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>R II</td>
<td>2.1</td>
<td>n.a.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R IV</td>
<td>1.0</td>
<td>n.a.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L II</td>
<td>1.3</td>
<td>-</td>
<td>(FP)</td>
</tr>
<tr>
<td></td>
<td>L III</td>
<td>2.0</td>
<td>-</td>
<td>(FP)</td>
</tr>
<tr>
<td></td>
<td>L IV</td>
<td>1.0</td>
<td>-</td>
<td>(FP)</td>
</tr>
<tr>
<td>10</td>
<td>L I</td>
<td>-</td>
<td>(TN)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>L II</td>
<td>1.6</td>
<td>-</td>
<td>(FP)</td>
</tr>
<tr>
<td></td>
<td>L III</td>
<td>1.3</td>
<td>-</td>
<td>(FP)</td>
</tr>
</tbody>
</table>
There were no false negative $^{18}$FLT-PET studies. Based on these findings, sensitivity of $^{18}$FLT for determining lymph node status in head and neck cancer patients was 100%, specificity 40%, positive predictive value 33.3% and negative predictive value 100% on lymph node level. On patient level, after excluding patient 7 as no histological correlate for the $^{18}$FLT-positive lymph node was available, numbers were 100%, 16.7%, 37.5% and 100%, respectively.

Not all lymph nodes showing enhanced $^{18}$FLT-PET-uptake were removed during surgery. These lymph nodes were either not included in the neck dissection specimen (patient 3 and 8), or situated in the contralateral neck (patient 1, 2, 4, 7, 9). Based on the results of the standard diagnostic work-up and therapeutic guidelines, there was no indication for removal of these nodes.

Follow-up (median 13 months, range 11-15 months) has revealed recurrent primary tumor in one patient treated with surgery and post-operative radiotherapy (pT2N0M0 carcinoma of the tongue). Until present, no lymph node recurrence has been observed in any of the patients.

### 7.3.4 Ki-67 and IdUrd staining

Ki-67 and IdUrd staining was present in metastatic tumor cells, germinal centers and in remaining lymphoid tissue as shown in Figure 7.2. In almost all lymph nodes examined, both $^{18}$FLT-PET positive and negative, germinal centers staining positive for Ki-67 and IdUrd were present. Metastatic tumor cells in patients 1 and 3 had almost fully destroyed the lymph node architecture. In patient 8, the micrometastasis occupied only a small region of the affected lymph node leaving reactive germinal centers and remaining lymph node tissue unperturbed.

In germinal centers, metastases, and remaining lymphoid tissue, nuclei staining positive for Ki-67 and IdUrd were counted and the LI was calculated. Figure 7.3 shows the overall results for Ki-67 LI and IdUrd LI in these three lymph node areas. Table 7.3 presents the quantitative data for Ki-67 LI and IdUrd LI in the germinal centers, remaining lymphoid tissue, metastases and overall for the true negative, false positive and true positive lymph nodes. The median Ki-67 and IdUrd LI in the germinal centers was 52.8% and 25.7% with no difference between the three groups. In the remaining lymphoid tissue this was 3.3% and 1.6% respectively, also with no difference between the groups. In the metastases, the median LI was 26.7% for Ki-67 and 9.3% for IdUrd. In all three areas and patient groups studied, Ki-67 LI was significantly higher compared to IdUrd LI ($p<0.0001$).
**Figure 7.3**  
*A: Ki-67 labeling index (LI) and B: IdUrd LI in germinal centers (GC), remaining lymphoid tissue (LT) and metastases (MET).*

**Table 7.3**  
*Ki-67 and IdUrd labeling index (LI) in germinal centers, remaining lymphoid tissue and in metastases*

<table>
<thead>
<tr>
<th>Ki-67 LI</th>
<th>germinal centers</th>
<th>remaining lymphoid tissue</th>
<th>metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TN* FP* TP† overall</td>
<td>TN FP TP overall</td>
<td>TP</td>
</tr>
<tr>
<td>mean</td>
<td>58.6 52.1 50.5 53.9</td>
<td>3.7 2.8 7.4 3.8</td>
<td>26.8</td>
</tr>
<tr>
<td>stdv§</td>
<td>16.6 13.2 4.2 13.8</td>
<td>1.9 2.4 7.5 3.6</td>
<td>7.7</td>
</tr>
<tr>
<td>median</td>
<td>55.1 53.1 49.5 52.8</td>
<td>3.6 2.5 4.6 3.3</td>
<td>26.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>IdUrd</th>
<th>germinal centers</th>
<th>remaining lymphoid tissue</th>
<th>metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TN FP TP overall</td>
<td>TN FP TP overall</td>
<td>TP</td>
</tr>
<tr>
<td>mean</td>
<td>33.5 27.4 23.0 28.8</td>
<td>1.4 1.8 4.2 2.1</td>
<td>10.4</td>
</tr>
<tr>
<td>stdv§</td>
<td>15.7 9.4 4.5 11.7</td>
<td>0.7 1.3 6.4 2.8</td>
<td>6.1</td>
</tr>
<tr>
<td>median</td>
<td>29.6 23.9 24.9 25.7</td>
<td>1.6 1.6 1.5 1.6</td>
<td>9.3</td>
</tr>
</tbody>
</table>

*TN = true negative (FLT negative lymph node without metastasis), FP = false positive (FLT positive lymph node without metastasis), TP = true positive (FLT positive lymph node with metastasis), §stdv = standard deviation

### 7.3.5 Germinal centers

The median number of germinal centers per lymph node was 9 (SD 6.7) in true negative lymph nodes, 20.5 (SD 27.0) in false positive nodes and 4 (SD 18.1) in true positive nodes. The difference in number of germinal centers between true negative and false positive lymph nodes was significant (p=0.03). Also the absolute area occupied by germinal centers was higher in false positive nodes relative to true negative nodes but the difference was only borderline significant (p=0.06), see Figure 7.4A. The total proliferative activity in the germinal centers expressed as Ki-67germinal center and IdUrdgerminal center was higher in the false positive lymph nodes compared to the true negative nodes, although the difference did not reach statistical significance (p=0.07, respectively), see Figures 7.4B and 7.4C.
Figure 7.4  
A: Absolute area (in mm²) occupied by germinal centers in true negative (TN; ^18^FLT negative lymph node without metastasis), false positive (FP; ^18^FLT positive lymph node without metastasis) and true positive (TP; ^18^FLT positive lymph node with metastasis) lymph nodes. B and C: Ki-67_germinai center and IdUrd_germinai center in TN and FP lymph nodes as a measure of the total proliferative activity in germinal centers (calculated as area in mm²).

7.3.6 Total proliferative activity in lymph nodes and correlation with SUV_{mean}  
The total proliferative activity in lymph nodes expressed as Ki-67_{lymph node} and IdUrd_{lymph node} was found to be significantly higher in the true positive lymph nodes as compared to false positive lymph nodes (p=0.006 and p=0.05, respectively). As shown in Figure 7.5, there was a moderate but significant correlation between SUV_{mean} and Ki-67_{lymph node} (r²=0.47, p=0.0009) and between SUV_{mean} and IdUrd_{lymph node} (r²=0.55, p=0.0004).

Figure 7.5  
A: Scatterplot of Ki-67_{lymph node} and B: scatterplot of IdUrd_{lymph node} as a measure of the total proliferative activity in the lymph node (calculated as area in mm²) versus SUV_{mean} of ^18^FLT-PET. Solid lines indicate linear best fit.
7.4 Discussion

The PET tracer $^{18}$FLT for imaging of cell proliferation has been studied by various groups, correlating $^{18}$FLT with $^{18}$FDG\textsuperscript{20-26,28-31}. Buck\textit{ et al.} studied $^{18}$FLT-PET and $^{18}$FDG-PET in 47 patients with benign and malignant pulmonary nodules and found a high sensitivity of FLT for malignant primary tumors (sensitivity 90%), but not for mediastinal lymph node involvement (sensitivity 53%) or detection of lung metastasis (sensitivity 67%)\textsuperscript{22}. Twenty-one patients with primary or recurrent laryngeal carcinomas were studied by Cobben\textit{ et al.}\textsuperscript{27}. Data on sensitivity and specificity for detection of the primary tumor were only reported for $^{18}$FDG and not for $^{18}$FLT. However, the standardized uptake value (SUV) for $^{18}$FLT was found to be significantly lower compared to the SUV for $^{18}$FDG and therefore the routine use of $^{18}$FLT-PET for detection of laryngeal carcinoma was not recommended. Additional studies correlated $^{18}$FLT with histological assessment of proliferation by Ki-67 labeling in fibrosarcoma, breast and lung cancer\textsuperscript{23,30-35}. In lung carcinoma, Buck\textit{ et al.} found that the Ki-67 LI correlated with $^{18}$FLT uptake\textsuperscript{23}. This finding has recently been confirmed by Yap\textit{ et al.}\textsuperscript{31}. In a fibrosarcoma xenograft, Leyton\textit{ et al.} found that $^{18}$FLT uptake and PCNA labeling index were linearly correlated\textsuperscript{33}. In contrast to these studies, Smyczek-Gargya\textit{ et al.} found no correlation between Ki-67 labeling index and $^{18}$FLT uptake in primary breast carcinoma\textsuperscript{30}. Although the results are not entirely consistent, these studies suggest that $^{18}$FLT-PET may be of value for the quantification of tumor cell proliferation.

In the current study, the value of $^{18}$FLT-PET in assessing the cervical lymph node status and proliferative activity of metastatic lymph nodes in squamous cell carcinoma of the head and neck was investigated. The endogenous proliferation marker Ki-67, which is expressed in the G\textsubscript{1}, S-, G\textsubscript{2}- and M-phase of the cell cycle, was chosen for comparison with $^{18}$FLT-PET, because endogenous markers do not require intravenous administration and because recent studies validated $^{18}$FLT-PET with Ki-67\textsuperscript{23,30,31}. In addition, the exogenous marker IdUrd was used. IdUrd is a robust and specific S-phase marker and it was hypothesized that this marker might correlate better with $^{18}$FLT uptake, because TK-1 activity is increased mainly during DNA synthesis. In nine of the ten patients studied, increased $^{18}$FLT uptake was observed in the lymph nodes. Only three of these nine $^{18}$FLT-PET positive patients had metastatic nodal disease confirmed by histopathology, and two of them had already been detected by routine preoperative screening. In none of the $^{18}$FLT-PET negative lymph nodes metastatic disease was present.

As the number of false positive lymph nodes was high, further analysis was performed taking into account the architecture and proliferative state of the lymph nodes. In most lymph nodes evaluated, intense staining of both Ki-67 and IdUrd was present in B-
lymphocytes proliferating in germinal centers. Less intense staining was found in metastatic tumor cell deposits and the proliferative activity in remaining lymph node tissue was very low. In germinal centers, the labeling index of both markers was significantly higher compared to metastases or lymphoid tissue. There was no difference in the proliferative activity in germinal centers between true negative and false positive lymph nodes. However, false positive nodes on average contained a significantly greater number of germinal centers and these occupied a larger absolute area relative to the true negative nodes, although this latter finding was only borderline statistically significant. Also the product of Ki-67 LI and IdUrd LI and area occupied by germinal centers was higher in the false positive compared to the true negative lymph nodes. It is therefore likely that the active proliferation of B-lymphocytes in germinal centers is responsible for the false-positive 18FLT-PET results. This high proliferative activity of B-lymphocytes might also be responsible for 18FLT-PET positivity of the micrometastasis in patient 8.

Three other studies, two in breast cancer and one in thoracic tumors compared 18FLT-PET with histopathology for the assessment of lymph node status. The study by Smyczek-Gargya et al. included 14 breast cancer patients, of whom eight had histologically proven axillary lymph node metastasis and seven were detected by 18FLT-PET (sensitivity 87.5% and specificity 100%) 30. In the study by Been et al. that included ten patients, only two of seven patients with histologically proven metastatic axillary lymph nodes were detected by 18FLT-PET (sensitivity 28.5% and specificity 100%) 20. Yap et al. studied 22 patients with thoracic tumors and reported sensitivity and specificity for detection of mediastinal lymph nodes by 18FLT to be 33.3% and 98.2%, respectively 31. The low sensitivity in some of these studies may be explained by the fact that some histological tumor types, such as mammary carcinoma, can exhibit limited proliferative activity. A second explanation might be that the metastatic tumor load of some of these lymph nodes was low.

All three studies reported a high specificity in contrast to the current study where specificity was only 16.7%. The localization of the lymph nodes may be of importance in explaining this discrepancy. Reactive lymph nodes in the head and neck area are found frequently as response to bacterial or viral infections, whereas reactive axillary lymph nodes are less common. Furthermore, patients with squamous cell carcinomas of the oral cavity – in this study the largest patient group – often present with non-healing ulcers accompanied with reactive lymph nodes. This is consistent with the observation that false positive lymph nodes on average contained a higher subvolume of germinal centers with very active proliferation of B-lymphocytes as compared to true negative nodes.
In this study, $^{18}$FLT-PET reached only low mean SUV in metastatic as well as in non-metastatic cervical lymph nodes compared to the SUV for $^{18}$FDG-PET generally reported in head and neck cancer \(^{6,8,10,28}\). This is in agreement with the previous finding for primary laryngeal tumors by Cobben et al. \(^{27}\). In accordance with studies discussed above, a significant correlation between SUV\(_{\text{mean}}\) and overall Ki-67 and IdUrd staining (Ki-67\(_{\text{lymph node}}\) and IdUrd\(_{\text{lymph node}}\)) was found in this study (Fig. 7.5) \(^{31,33}\). The correlation was strongest for IdUrd and we recommend this marker for future studies with $^{18}$FLT that include histological validation.

### 7.5 Conclusions

Although $^{18}$FLT-PET correctly identified all head and neck cancer patients with metastatic lymph nodes, the specificity and positive predictive value were low due to tracer-uptake in germinal centers of lymph nodes. Therefore, the use of $^{18}$FLT-PET for assessing pretreatment lymph node status and for determining the proliferative activity of affected lymph nodes is not encouraged in head and neck cancer patients. This also limits the usefulness of $^{18}$FLT-PET for radiation therapy planning and possibly for early treatment response evaluation of small metastatic lymph nodes. The value of $^{18}$FLT-PET for assessment of the proliferative state of the primary head and neck tumor and the relevance for radiotherapy planning is a topic of current investigations.

### Acknowledgements

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### 7.6 References

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18FLT-PET for detection of lymph node metastases


Chapter 8

$^{18}$FLT-PET-CT for early response monitoring and dose escalation in oropharyngeal tumors

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Otto C. Boerman
Wim J.G. Oyen
Johannes H.A.M. Kaanders

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Abstract

Introduction. Accelerated tumor cell proliferation is an important mechanism adversely affecting therapeutic outcome in head and neck cancer. 3′-deoxy-3′-18F-fluorothymidine (18FLT) is a PET tracer to non-invasively image tumor cell proliferation. The aims of this study were to monitor early tumor response based on repetitive 18FLT-PET-CT scans and to identify subvolumes with high proliferative activity eligible for dose escalation.

Materials and Methods. Ten patients with oropharyngeal tumors underwent an 18FLT-PET-CT scan prior to and twice during radiotherapy. The primary tumor and metastatic lymph nodes were delineated based on CT (GTVct), and after segmentation of the PET signal using the 50% isocontour of the maximum signal intensity (GTV50%) or an adaptive threshold based on the signal-to-background ratio (GTVsbr). Gross tumor volumes were calculated, and similarity between GTVct and GTVsbr was assessed. Within GTVsbr, the maximum and mean standardized uptake values (SUVmax and SUVmean) were calculated. Within GTVct, tumor subvolumes with high proliferative activity were identified based on the 80% isocontour (GTV80%) for radiotherapy planning with dose escalation.

Results. The GTVct decreased significantly in the fourth week, but not in the initial phase of treatment. SUVmax and SUVmean decreased significantly as early as one week after therapy initiation and even further before the fourth week of treatment. For the primary tumor, the average (± 1 SD) SUVmean values of the GTVsbr were 4.7 (± 1.6), 2.0 (± 0.9) and 1.3 (± 0.2) for the consecutive scans (p<0.0001). The similarity between GTVct and GTVsbr decreased during treatment, indicating an enlargement of GTVsbr outside GTVct caused by increasing difficulty to segment tracer uptake in the tumor from the background, and by proliferative activity in the nearby tonsillar tissue. Subvolumes with high proliferative activity (GTV80%) were successfully identified in all primary tumors and metastatic lymph nodes, and dose escalation based on the GTV80% was demonstrated to be technically feasible.

Conclusions. 18FLT is a promising PET tracer for imaging tumor cell proliferation in head and neck carcinomas. 18FLT-PET signal changes precede volumetric tumor response and are therefore suitable for early response assessment. Definition of tumor subvolumes with high proliferative activity and dose escalation to these regions is technically feasible.
8.1 Introduction

Radiotherapy with or without concomitant chemotherapy is the therapy of choice for advanced stage primary squamous cell carcinomas of the oropharynx. For radiation therapy planning, a single computed tomography (CT) scan in treatment position is acquired prior to the initiation of treatment. Traditionally, a homogeneous dose distribution is prescribed to the gross tumor volume (GTV) defined on CT with a margin for subclinical growth and setup inaccuracy. In the last decade, intensity modulated radiation therapy (IMRT) revolutionized the field of radiotherapy. IMRT is based on the use of photon beams with optimized non-uniform fluence profiles. With this technique, the simultaneous delivery of different dose prescriptions to various target (sub)sites became feasible. Certain areas within the GTV can since be boosted to higher doses, whilst steep dose gradients allow to reduce the dose delivered to radiation sensitive tissues adjacent to the tumor. For this means, however, precise knowledge about the tumor location and extension is compulsory, and tumor areas requiring higher radiation doses must be identified.

Functional imaging can complement anatomical imaging modalities such as CT and magnetic resonance imaging (MRI) and provide biological tumor information relevant for radiotherapy dose planning. Initial studies with positron emission tomography (PET) using 18F-fluorodeoxyglucose (18FDG) demonstrated that the volume irradiated to high-dose levels can be reduced, thus promoting normal structure sparing and dose escalation. 18FDG uptake reflects metabolic activity, and false positive readings are caused by tracer uptake in inflammatory tissue. Other PET tracers can provide more specific biological information, in particular on radiotherapy resistance mechanisms.

Accelerated tumor cell repopulation during the course of radiotherapy is a frequently observed phenomenon in squamous cell carcinomas of the head and neck that adversely affects treatment outcome. 3'-deoxy-3'-18F-fluorothymidine (18FLT) uptake is enhanced during DNA synthesis, and 18FLT-PET therefore provides a non-invasive imaging modality for tumor cell proliferation. Potential applications of 18FLT-PET in radiation oncology are: patient selection for treatment modification based on the tumor's proliferative state, identification of tumor subvolumes with a high density of actively proliferating cells amenable for boosting, and early treatment response monitoring.

In this study, patients with oropharyngeal tumors underwent three subsequent 18FLT-PET scans: prior to and twice during the course of radiotherapy. The aims of this study were: 1) to monitor early tumor response based on 18FLT-PET-CT volume and 18FLT-PET signal changes, 2) to assess the heterogeneity of intratumoral 18FLT distribution and identify subvolumes with a high density of proliferating cells, and 3) to determine the technical feasibility of adaptive radiation therapy based on 18FLT-PET-CT.
8.2 Material and Methods

Patients. From March 2007 until September 2008, 10 patients with newly diagnosed primary oropharyngeal carcinomas eligible for radiotherapy with or without concomitant chemotherapy were included in this study after giving written informed consent. The Institutional Review Board of the Radboud University Nijmegen Medical Centre approved the study.

Treatment. Patients were treated with intensity modulated radiotherapy with a simultaneous integrated boost (SIB) technique, delivering a dose of 68 Gy in fractions of 2 Gy to the primary tumor and metastatic cervical lymph nodes and 50.3 Gy in fractions of 1.48 Gy to the electively treated neck nodes. An accelerated fractionation schedule was used with an overall treatment time of 5.5 weeks delivering two fractions per day during the last 1.5 weeks of the treatment. Following the institution’s guidelines, two patients with bulky primary tumors were concomitantly treated with cisplatinum 40 mg/m², administered intravenously once weekly.

18FLT synthesis. 18FLT was obtained from the Department of Nuclear Medicine and PET Research, VU University Medical Centre, Amsterdam, the Netherlands. The synthesis was performed according to the method of Machulla et al. Briefly, 18FLT was produced by 18F-fluorination of the 4,4'-dimethoxytrityl protected anhydrothymidine, followed by a deprotection step. The product was purified by reversed phase high-performance liquid chromatography, made isotonic and passed through a 0.22 μm filter. 18FLT was routinely produced with a non-decay corrected radiochemical yield of 5-10%, a radiochemical purity of >97% and a specific activity higher than 10,000 GBq/mmol.

18FLT-PET-CT acquisition. Prior to and in the second and fourth weeks of radiotherapy, integrated 18FLT-PET and CT images were acquired on a hybrid PET-CT scanner (Siemens Biograph Duo scanner; Siemens/CTI, Knoxville, TN, USA). All scans were performed with the patient in supine position and immobilized with an individual head support and a rigid customized mask covering the head and neck area in order to increase position accuracy and to reduce movement artifacts during image acquisition. Emission images of the head and neck area were recorded 60 minutes after intravenous injection of approximately 250 MBq 18FLT, with 7 minutes per bed position in 3D mode. PET images were reconstructed using the OSEM iterative algorithm with parameters optimized for the head and neck area (i.e., 4 iterations, 16 subsets and 5 mm 3D Gaussian filter), with correction for photon attenuation. In addition, CT images were acquired for anatomical correlation and attenuation correction purposes using 80 mAs, 130 kV, 3 mm slice width, and intravenous contrast in the venous phase (Optiray, Mallinckrodt Inc., Hazelwood, MO, USA).
**18FLT-PET analysis.** After reconstruction, SUV-PET images were created with in-house developed software correcting for injected dose, decay of the tracer, and patient body weight. Subsequently, these SUV-PET images were resliced using the CT format as reference. SUV-PET and CT images were imported into Pinnacle³ (version 8.0d; Philips Radiation Oncology System, Madison, WI, USA), the radiotherapy planning system routinely used at our department. With this software, the consecutive CT and PET scans were registered to the first CT-scan using cross-correlation. Two investigators delineated the gross tumor volume (GTV) of the primary tumor and the metastatic lymph nodes on all registered CT scans (GTV\(\text{CT}\)). Self-written scripts in Pinnacle³ were used for the segmentation of the primary tumor and the metastatic lymph nodes from the PET images and for the calculation of the mean and maximum standardized uptake value (SUV\(_{\text{mean}}\) and SUV\(_{\text{max}}\)) within these volumes.

**PET segmentation of primary tumor and metastatic lymph nodes.** Two previously described methods for segmentation of the primary tumor and the metastatic lymph nodes in the PET images were applied \(^7, 18\). Firstly, the 50\% isocontour (GTV\(_{50\%}\)) was based on a fixed percentage of the maximum signal intensity in the primary tumor. Secondly, an adaptive threshold delineation based on the signal-to-background ratio was performed (GTV\(_{\text{SBR}}\); Fig. 8.1) \(^18\).

For this means, the SUV\(_{\text{max}}\) was defined as mean uptake of the hottest voxel in the tumor or metastatic lymph node and its eight surrounding voxels in one transversal slice. Mean background uptake was calculated from a manually defined region of interest in the left neck musculature (approximately 10 cm\(^3\)) at sufficient distance from the vertebrae, the primary tumor and lymph node metastases.

**PET segmentation of subvolumes with high proliferative activity.** Based on the \(^18\)FLT-PET signal, tumor subvolumes with a high density of proliferating tumor cells within the GTV\(_{\text{CT}}\) of the primary tumor and metastatic lymph nodes were defined. An arbitrary, fixed threshold of the SUV\(_{\text{max}}\) was defined that fulfilled the requirement of delineating a tumor subvolume in at least the first and second \(^18\)FLT-PET scan. This was best met using the 80\% isocontour (GTV\(_{80\%}\)). The absolute GTV\(_{80\%}\) and the fraction of the GTV\(_{80\%}\) relative to the GTV\(_{\text{CT}}\) were calculated. The GTV\(_{80\%}\) from the third scan was not further analyzed, because of a very low \(^18\)FLT-uptake in the tumor and thus a relatively low SUV\(_{\text{max}}\) value within the GTV\(_{\text{CT}}\). This lead to unsuccessful segmentation of PET subvolumes, which were mostly larger than the GTV\(_{\text{CT}}\) and often encompassed the entire tonsillar region.

**Volumetric and spatial similarity.** The absolute volumetric similarity of the gross tumor volume on CT (GTV\(_{\text{CT}}\)) and \(^18\)FLT-PET (GTV\(_{\text{SBR}}\)) was assessed at all three time points. For this analysis only GTV\(_{\text{SBR}}\) was used. As it does not adjust for background
activity, segmentation of the tumor by GTV_{50\%} became increasingly difficult with lower $^{18}$FLT-uptake in the second and third scans.

As measures of volumetric similarity, we calculated the part of the GTV_{CT} that was not covered by the GTV_{SBR} ($\text{simGTV}_{CT}$) and vice versa: the GTV_{SBR} not enclosed by the GTV_{CT} ($\text{simGTV}_{SBR}$). As a measure reflecting differences in location more strongly than differences in size, the Dice Similarity Coefficient (DSC) was additionally calculated as twice the overlap volume divided by the sum of both volumes:

$$\text{DSC} = 2 \times (\text{GTV}_{CT} \cap \text{GTV}_{SBR}) / (\text{GTV}_{CT} + \text{GTV}_{SBR})$$

It is generally accepted that a value of DSC > 0.7 represents excellent agreement. The same calculations were applied to the two GTV_{80\%} delineated on the first and second $^{18}$FLT-PET scans, GTV_{80\%1} and GTV_{80\%2}.

**Statistical analysis.** Statistical analyses were performed using GraphPad Prism Version 4.0c for Macintosh (La Jolla, CA). Gaussian distribution was tested using the Kolmogorov-Smirnov test. The change in CT and PET volumes was assessed using repeated measures ANOVA and two-tailed paired t-test, and the Friedman test and Wilcoxon signed rank test, respectively. The alteration in PET signal intensity was assessed using two-tailed paired t-test. The volumetric similarity of the GTVs delineated on CT and PET was analyzed using Friedman test and Wilcoxon signed rank test. The repeated measures ANOVA and two-tailed paired t-test were applied to the Dice Similarity Coefficient. The volumetric change in GTV_{80\%} was assessed using the Wilcoxon signed rank test. A p-value < 0.05 was regarded statistically significant.

### 8.3 Results

#### 8.3.1 Patients and tumor characteristics

The patient and tumor characteristics are summarized in Table 8.1. Eight patients were treated with radiotherapy alone, two patients with the addition of concomitant chemotherapy. The first $^{18}$FLT-PET scan was acquired before the start of therapy (median 5 days, range 0 – 9 days). The second and third scan were acquired in the second and fourth week of treatment (see Table 8.1 for details). All primary tumors and lymph node metastases were visualized and subject to further assessment.

#### 8.3.2 Early response monitoring: Reduction of tumor volume

The mean primary tumor volume delineated on CT (GTV_{CT}) decreased significantly between the second and third CT-scan, but not in the initial phase of treatment: mean GTV_{CT} ($\pm$ 1 SD) on subsequent scans 12.7 cm$^3$ ($\pm$ 9.5 cm$^3$), 11.1 cm$^3$ ($\pm$ 8.8 cm$^3$) and 5.0 cm$^3$ ($\pm$ 4.7 cm$^3$) (Fig. 8.2A).

Although the overall $^{18}$FLT signal intensity decreased significantly after the start of treatment, the segmented PET volume remained almost unchanged when the SBR method was used: mean GTV_{SBR}: 11.8 cm$^3$ ($\pm$ 8.9 cm$^3$), 11.3 cm$^3$ ($\pm$ 12.4 cm$^3$) and
14.1 cm³ (± 10.8 cm³) (Fig. 8.2A), and even increased when the 50% threshold of the maximum signal intensity was applied: mean GTV$_{50\%}$ of 18.0 cm³ (± 31.5 cm³), 18.6 cm³ (± 21.8 cm³) and 40.9 cm³ (± 61.1 cm³). For the two patients treated with concomitant radiotherapy and chemotherapy, the decrease in $^{18}$FLT-PET signal was similar to that of the patients treated with radiotherapy alone. For GTV delineation of the lymph nodes the trends were similar as for the primary tumors, i.e., a decrease in GTV$_{ct}$ by the end of the treatment and no significant change in GTV$_{sbr}$ (Fig. 8.2B). As the 50% method resulted in unsatisfactory results in the primary tumors, it was not further applied to the metastatic lymph nodes.

### Table 8.1 Patient characteristics

<table>
<thead>
<tr>
<th>Patient</th>
<th>Site</th>
<th>Gender</th>
<th>Age</th>
<th>Clinical stage</th>
<th>Treatment</th>
<th>Cumulative dose delivered at PET2 (Gy)</th>
<th>Cumulative dose delivered at PET3 (Gy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tonsil</td>
<td>f</td>
<td>67</td>
<td>T3N0M0</td>
<td>RCHT</td>
<td>16</td>
<td>36</td>
</tr>
<tr>
<td>2</td>
<td>Soft palate</td>
<td>m</td>
<td>66</td>
<td>T2N1M0</td>
<td>RCHT</td>
<td>16</td>
<td>36</td>
</tr>
<tr>
<td>3</td>
<td>Base of tongue / tonsil</td>
<td>m</td>
<td>60</td>
<td>T2N0M0</td>
<td>RT</td>
<td>12</td>
<td>36</td>
</tr>
<tr>
<td>4</td>
<td>Tonsil</td>
<td>m</td>
<td>53</td>
<td>T2N2bM0</td>
<td>RT</td>
<td>16</td>
<td>36</td>
</tr>
<tr>
<td>5</td>
<td>Base of tongue / tonsil</td>
<td>m</td>
<td>69</td>
<td>T2N0M0</td>
<td>RT</td>
<td>12</td>
<td>32</td>
</tr>
<tr>
<td>6</td>
<td>Tonsil</td>
<td>f</td>
<td>53</td>
<td>T3N0M0</td>
<td>RT</td>
<td>24</td>
<td>36</td>
</tr>
<tr>
<td>7</td>
<td>Tonsil / hypopharynx</td>
<td>m</td>
<td>64</td>
<td>T2N1M0</td>
<td>RT</td>
<td>16</td>
<td>36</td>
</tr>
<tr>
<td>8</td>
<td>Tonsil</td>
<td>m</td>
<td>55</td>
<td>T2N2bM0</td>
<td>RT</td>
<td>20</td>
<td>36</td>
</tr>
<tr>
<td>9</td>
<td>Tonsil</td>
<td>m</td>
<td>70</td>
<td>T2N0M0</td>
<td>RT</td>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>10</td>
<td>Tonsil</td>
<td>m</td>
<td>52</td>
<td>T1N2bM0</td>
<td>RT</td>
<td>12</td>
<td>36</td>
</tr>
</tbody>
</table>

f = female; m = male; RT = radiotherapy; RCHT = radiotherapy + chemotherapy.

### 8.3.3 Early response monitoring: Decrease of standardized uptake value

In the primary tumors, the SUV$_{\text{max}}$ of the second $^{18}$FLT-PET-scan was already significantly decreased relative to the first scan, and even further in the third [mean SUV$_{\text{max}}$ (± 1 SD): 7.6 (± 2.6), 3.1 (± 1.7) and 1.7 (± 0.4); Fig. 8.2C]. The same was observed for SUV$_{\text{mean}}$ within GTV$_{\text{sbr}}$ [mean SUV$_{\text{mean}}$: 4.7 (± 1.6), 2.0 (± 0.9) and 1.3 (± 0.2); Fig. 8.2C]. However, on an individual patient basis, different response patterns became apparent (Fig. 8.2E). On average, the relative decrease in SUV$_{\text{max}}$ was 55% between the first and second, and 34% between the second and third $^{18}$FLT-PET scan.

In the lymph node metastases, similar patterns were observed for SUV$_{\text{max}}$ and SUV$_{\text{mean}}$ (Fig. 8.2D). The relative decrease in SUV$_{\text{max}}$ between the first and second $^{18}$FLT-PET scan was 52% and 36% respectively.
scan was 44%, and between the second and third 47%, again with individual differences (Fig. 8.2F).

Figure 8.1  
\[^{18}\text{FLT-PET-CT} \text{ images of a T3N0M0 oropharyngeal tumor obtained prior to radiation therapy. The gross tumor volume is delineated on CT (GTV}_{\text{ct}}; \text{ red), and after segmentation of the PET image using the signal-to-background method (GTV}_{\text{sbr}}; \text{ green) and the 50\% isocontour (GTV}_{50\%}; \text{ yellow). The subvolume of highest proliferative activity segmented on the PET image using the 80\% isocontour (GTV}_{80\%}) \text{ is highlighted in pink.} \]

8.3.4 Similarity of CT and \(^{18}\text{FLT-PET volume before and during treatment} \)

In order to assess the feasibility of adaptive radiotherapy based on repetitive \(^{18}\text{FLT-PET-CT} \) scanning, the absolute volumetric similarity and Dice Similarity Coefficient (DSC) of the primary gross tumor volumes delineated on CT (GTV\text{CT}) and PET (GTV\text{sbr}) were assessed at all time points.

There was a significant decrease in the absolute GTV\text{CT} volume not covered by GTV\text{sbr} at the third time point (Fig. 8.3A) indicating an almost complete coverage of the GTV\text{CT} by the GTV\text{sbr}. On the other hand, there was a significant increase in the GTV\text{sbr} volume not enclosed by GTV\text{CT} indicating a large GTV\text{sbr} outside the GTV\text{CT} that even increased over time (Fig. 8.3A). This decrease in volumetric and especially in spatial concordance was reflected by a significant decline of the DSC at the second and third PET scan (Fig. 8.3B).

The mean DSC (± 1 SD) was: 0.71 (± 0.08), 0.55 (± 0.21) and 0.29 (± 0.16) on the consecutive scans. This decrease in concordance was partly caused by increasing tracer
uptake in the tonsillar region, especially in the fourth week of treatment, and partly by low tracer uptake in the primary tumor hampering the adaptive threshold method.

Figure 8.2  
A and B: Gross tumor volume (in cm$^3$) delineated on CT (GTV$_{CT}$) and on PET using the signal-to-background method (GTV$_{SBR}$) for the primary tumor (A) and metastatic lymph nodes (B). C and D: Maximum and mean standardized uptake value (SUV$_{max}$ and SUV$_{mean}$) analyzed using the SBR method for the primary tumor (C) and the metastatic lymph nodes (D). The volumes and standardized uptake values for the first (green), second (yellow) and third (blue) scans are shown. n.s. = not significant, *p < 0.01, **p < 0.001, ***p < 0.0001. E and F: Alteration in SUV$_{max}$ on an individual patient basis for all primary tumors (E) and the metastatic lymph nodes (F). Each patient with cervical lymph node metastases is highlighted by an identical color code.
8.3.5 Delineation of subvolume with high density of proliferating cells using $^{18}$FLT-PET

For all primary tumors, a GTV$_{80\%}$ within the GTV$_{CT}$ could be identified on the first and second $^{18}$FLT-PET-CT scan (Fig. 8.4). In the third scan, this was hampered by a relatively low $^{18}$FLT uptake in the tumor relative to the background, leading to unsuccessful segmentation and subsequently to PET subvolumes larger than the GTV$_{CT}$, often encompassing the entire tonsillar region. The average GTV$_{80\%}$ ($\pm$ 1 SD) decreased slightly between the first and second scan [1.52 cm$^3$ ($\pm$ 1.76 cm$^3$) and 0.93 cm$^3$ ($\pm$ 0.94 cm$^3$), respectively]. Compared to the GTV$_{CT}$, the GTV$_{80\%}$ was relatively small and the fraction GTV$_{80\%}$/GTV$_{CT}$ overall did not change during treatment [prior to treatment: 0.12 ($\pm$ 0.07); second week of treatment: 0.12 ($\pm$ 0.14)]. On an individual patient basis, however, remarkable changes occurred in some cases, e.g., patients 1, 7 and 8 (Fig. 8.4). Between the first and second scan, the GTV$_{80\%}$ decreased and displaced (patient 1 and 7), or substantially increased (patient 8).

In order to assess these changes in size and location of the GTV$_{80\%}$, the absolute volumetric similarity and DSC of GTV$_{80\%1}$ and GTV$_{80\%2}$ ($\pm$ 1 SD) were calculated. The average volume of the GTV$_{80\%1}$ not covered by GTV$_{80\%2}$ was 1.12 cm$^3$ ($\pm$ 1.67 cm$^3$) and
the average volume of the GTV\textsubscript{80\%1} not encompassed by GTV\textsubscript{80\%1} was 0.56 cm\textsuperscript{3} (± 0.86 cm\textsuperscript{3}). The mean overall DSC was 0.47 (± 0.25), indicating a moderate spatial similarity between the first and second tumor subvolume. However, there were large inter-individual differences, reflected by a range in DSC from 0.03 to 0.86. For all lymph node metastases, a subvolume with a high density of proliferating cells could also be identified. The mean absolute overall volume did not significantly change between the first and second scan \[1.20 \text{ cm}^3 (± 1.13 \text{ cm}^3)\] and \[1.21 \text{ cm}^3 (± 1.25 \text{ cm}^3),\] respectively, nor did the mean fraction of GTV\textsubscript{80\%} compared to the GTV\textsubscript{ct} [prior to treatment: 0.17 (± 0.08); second week of treatment: 0.19 (± 0.14)].

**8.3.6 Generation of IMRT plan with boost to \textsuperscript{18}FLT-PET delineated subvolumes**

As a proof of principle, an adaptive IMRT treatment plan with SIB and an accelerated schedule was generated for a patient with a T3N0M0 oropharyngeal tumor. This plan was based on the CT scan acquired prior to treatment taking into account changes in proliferative activity occurring during treatment (Fig. 8.5).

With this technique, the neck was treated bilaterally to a dose of 50.3 Gy in fractions of 1.48 Gy, and the gross tumor volume delineated on CT (GTV\textsubscript{ct}) with margin for subclinical spread and setup inaccuracy was irradiated to a total dose of 68 Gy in 2 Gy fractions. The primary tumor subvolume with highest density of proliferating cells was defined twice: based on the \textsuperscript{18}FLT-PET scan acquired before the initiation of treatment (GTV\textsubscript{80\%1}) and on the scan obtained in the second week (GTV\textsubscript{80\%2}). Subsequently, the GTV\textsubscript{80\%1} was defined as boost subvolume for the first two weeks of treatment, and the GTV\textsubscript{80\%2} for dose escalation during weeks three and four. From week five, no increased dose was delivered to a highly proliferative subvolume. The GTV\textsubscript{80\%1} and GTV\textsubscript{80\%2} were consecutively irradiated in fractions of 2.3 Gy using the SIB technique resulting in a total dose of 74 Gy to the overlapping volume of the GTV\textsubscript{80\%1} and GTV\textsubscript{80\%2}, and a total dose of 71 Gy to the areas of GTV\textsubscript{80\%1} and GTV\textsubscript{80\%2} mismatch. The volume irradiated to a higher dose was small and neither impacted on the dose to the electively treated volume and primary tumor volume (PTV> and PTV<, respectively), nor to the dose to normal tissues (Table 8.2).
Figure 8.4  Detail of CT scans showing the primary oropharyngeal tumors (for numerical order see Table 8.1). Delineation of the gross tumor volume defined on CT ($GTV_{\text{CT}}$: first CT scan: red, second CT scan: orange) and of the subvolume with highest proliferative activity defined on $^{18}$FLT-PET ($GTV_{90\%}$: first PET scan: green, second PET scan: skyblue) is shown.
Figure 8.5  Dose escalation to the tumor subvolumes with highest proliferative activity (GTV_{80\%} and GTV_{90\%}) for a T3N0M0 oropharyngeal tumor. Using intensity modulated radiotherapy with integrated simultaneous boost technique, the total dose to the bilateral cervical lymph nodes regions was 50.3 Gy (large planning target volume, PTV; red) and to the primary tumor 68 Gy (small PTV; blue). The GTV_{80\%} (black) and GTV_{90\%} (green) were consecutively irradiated with 2.3 Gy for 10 fractions resulting in a total dose of 71 Gy and a dose of 74 Gy in the overlapping region. A: Dose distribution for first two weeks of treatment, and B: dose distribution for week three and four; see legend 1. C: Dose distribution for remaining 14 fractions without dose escalation; see legend 2. Dose distribution of total treatment plan in transverse (D) and sagittal (E) plane; see legend 3. The parotid glands are delineated in skyblue and the spinal cord in green.
### Table 8.2

Comparison of SIB-IMRT dose distributions: classical versus $^{18}$FLT-PET guided dose escalation

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Classical</th>
<th>$^{18}$FLT-PET guided dose escalation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organs at risk</td>
<td></td>
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</tr>
<tr>
<td>Left parotid gland (Gy)</td>
<td>25.4</td>
<td>25.4</td>
</tr>
<tr>
<td>Right parotid gland (Gy)</td>
<td>24.8</td>
<td>24.8</td>
</tr>
<tr>
<td>$D_{\text{max}}$ spinal cord (Gy)</td>
<td>48.2</td>
<td>48.1</td>
</tr>
<tr>
<td><strong>PTV&gt;</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$D_{\text{mean}}$ (Gy)</td>
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<td>52.1</td>
</tr>
<tr>
<td>$D_{90}$ (%)</td>
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</tr>
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<td></td>
</tr>
<tr>
<td>$D_{\text{mean}}$ (Gy)</td>
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</tr>
<tr>
<td>$D_{90}$ (%)</td>
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</tr>
<tr>
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<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>$D_{\text{mean}, \text{1st subvolume}}$ (Gy)</td>
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<td>73.6</td>
</tr>
<tr>
<td>$D_{\text{mean}, \text{2nd subvolume}}$ (Gy)</td>
<td>n.a.</td>
<td>74.5</td>
</tr>
<tr>
<td>$D_{\text{mean}, \text{overlap subvolumes}}$ (Gy)</td>
<td>n.a.</td>
<td>74.7</td>
</tr>
</tbody>
</table>

$D_{\text{max}}$ = maximum dose; $D_{\text{mean}}$ = mean dose; $D_{90}$ = percentage of volume receiving 90% of the prescribed dose; $D_{95}$ = percentage of volume receiving 95% of the prescribed dose; PTV> = planning target volume (PTV) encompassing electively treated volume, i.e., lymph node regions at risk of sub-clinical metastatic disease; PTV< = PTV encompassing primary tumor; PTV<< = PTV for dose escalation defined by repetitive $^{18}$FLT-PET imaging (see Methods and Materials); n.a. = not available.

### 8.4 Discussion

**Early response measurement**

Accelerated tumor cell proliferation is an important mechanism of treatment failure in head and neck cancer. A prerequisite for monitoring and early adaptation of treatments that counteract this resistance mechanism is visualization and quantification of the proliferating tumor cell compartment before and during treatment.

$^{18}$FLT is the most widely used PET tracer for imaging tumor proliferation. Alterations in the standardized uptake value of $^{18}$FLT have been used for early response monitoring in a variety of human tumors. Wieder et al. studied rectal cancer patients prior to treatment, 2 weeks after initiation and 3-4 weeks after completion of treatment. Although the SUV$_{\text{mean}}$ decreased significantly 14 days after the start of chemoradiation, this did not correlate with histopathological tumor regression. Herrmann et al. assessed two groups of patients with non-Hodgkin's lymphoma undergoing chemotherapy and found $^{18}$FLT SUV$_{\text{max}}$ to already decrease significantly after 2 days. Furthermore, the authors were able to detect a significant difference in reduction of tumoral $^{18}$FLT uptake.
between patients reaching partial response versus complete response at the end of therapy. Finally, two groups studied $^{18}$FLT uptake in breast cancer patients. Kenny et al. examined patients prior to and one week after neoadjuvant chemotherapy and found the $^{18}$FLT SUV to decrease significantly discriminating between patients with clinical response or stable disease. Pio et al. found changes in $^{18}$FLT uptake after one course of chemotherapy to correlate significantly with changes in tumor size detected by CT. Recently, Menda et al. demonstrated in eight head and neck cancer patients that initial $^{18}$FLT uptake and change early after treatment can be adequately monitored by SUV obtained at 45-60 min. Furthermore, a study on 15 patients (including six head and neck cancer patients) provided evidence that changes in $^{18}$FLT-PET SUV$_{\text{max}}$ of more than 20-25% are likely to be therapy-related. Thus, under the assumption that repetitive quantitative $^{18}$FLT-PET measurements are sufficiently reproducible, $^{18}$FLT-uptake quantified by SUV has the potential to monitor changes in the proliferative activity of tumors early during treatment.

Based on these assumptions, our study assessed early response in patients with oropharyngeal tumors treated with radiotherapy or chemoradiation. Overall, there was a significant treatment-induced decrease in $^{18}$FLT tracer uptake, both in the primary tumor and cervical lymph node metastases as early as after the fifth fraction of radiotherapy. This was reflected by a greater than two-fold reduction in standardized uptake values in the initial phase of treatment and a further two-fold reduction in the fourth week. Clearly, different individual response patterns were also found in this study. Some patients showed a moderate decrease in standardized uptake values, whilst others even responded with an increase in signal intensity. Longer follow-up and a larger patient cohort are necessary in order to assess whether these individual differences will be discriminative for ultimate tumor response.

One could hypothesize that the concomitant use of chemotherapy may reduce tumor cell proliferation more rapidly compared to radiotherapy alone. However, the two patients treated with chemoradiotherapy in this study did not show a more rapid decrease in $^{18}$FLT-uptake compared to the others. We will further address this issue in a subsequent study including a larger number of patients treated with chemotherapy. Results of a meta-analysis indicate a survival benefit of concomitant chemotherapy and radiotherapy and this has become the standard of care for advanced head and neck cancer. It is therefore of importance that also the effects of chemotherapy on tumor cell proliferation be investigated in future early response studies.

**Adaptive radiotherapy based on $^{18}$FLT-PET-CT**

In current practice, radiotherapy planning is based on a single CT-scan acquired prior to the start of treatment. During treatment, however, the tumor and metastatic lymph nodes shrink, and the patient may loose weight. As a result, the dose distribution in the
tumor and organs at risk may change. In addition, as demonstrated in this study, biological aspects of the tumor can change even more rapidly and dramatically. Adaptation of the target volume based on CT or on functional imaging is a means to correct for these treatment-induced alterations. Amongst other PET-radiopharmaceuticals, $^{18}$FLT is one of the potential tracers for adaptive radiotherapy as it specifically visualizes one of the tumor characteristics responsible for treatment failure.

In contrast to the early treatment-associated decrease in $^{18}$FLT uptake, significant changes in gross tumor volume on CT were only detectable in the fourth week (after 15 to 18 fractions). Analyses of volumetric changes based on $^{18}$FLT-PET, however, were not successful, possibly due to limitations of the applied PET segmentation techniques. During treatment, the signal intensity within the tumor decreased relative to the background. This observation might argue against the generally accepted phenomenon of accelerated tumor cell repopulation during a course of fractionated radiotherapy. However, the decrease in $^{18}$FLT-PET signal is caused mainly by a rapid reduction of the tumor cell density as a result of the treatment. This does not exclude the possibility that the relative number of proliferating tumor cells, i.e., the proliferating fraction, is nevertheless increasing. At the same time, $^{18}$FLT uptake in the tonsillar region increased most likely caused by proliferating inflammatory cells. These two phenomena hampered PET segmentation based on the fixed threshold of 50%. The adaptive threshold delineation based on the signal-to-background ratio performed somewhat better, but also failed at the third time point when $^{18}$FLT-uptake was very low. As a consequence, $^{18}$FLT-PET was neither useful for volumetric response monitoring, nor for the adaptation of gross tumor volume delineation during treatment. New iterative methods are becoming available, but it is questionable whether these can overcome these limitations. The disturbance by increased proliferative activity in the tonsillar tissue may be a lesser problem in other head and neck subsites.

**Dose escalation to highly proliferative subvolumes**

PET may potentially identify parts of the tumor requiring additional radiation doses, e.g., areas of high metabolic or proliferative activity, or hypoxic subvolumes. In this study, $^{18}$FLT-PET was successfully used to identify subvolumes with high proliferative activity before and in the second week of therapy in all primary tumors and metastatic lymph nodes. It was shown that in a number of patients the subvolumes changed during the initial phase of treatment with respect to size and location. Therefore, repetitive imaging for proper monitoring of the high proliferative subvolumes is necessary. Furthermore, image acquisition at a late time point during treatment, e.g., the fourth week, does not lead to useful results due to very low signal intensities. Similar findings on temporal changes in hypoxic subvolumes have previously been described, even without any treatment. Lin et al. studied seven head and neck cancer patients
undergoing serial $^{18}$F-fluoromisonidazole ($^{18}$FMISO) PET scans, separated by 3 days, prior to the start of treatment. In four of these patients, significant dissimilarities in the hypoxic subvolumes were observed within this short time window.

Various theoretical planning studies applying either uniform dose distribution, dose painting or voxel intensity-based IMRT to $^{18}$FDG or $^{18}$FMISO-PET avid subvolumes have been published $^8,^9,^{31-34}$. Schwartz et al. escalated the total dose up to 75 Gy in a theoretical planning study involving 20 head and neck cancer patients $^8$. Rajendran et al. demonstrated that using an IMRT technique, the dose to the $^{18}$FMISO-PET detected hypoxic subvolume could be escalated by an additional 10 Gy, and Lee et al. even achieved a dose of 84 Gy in hypoxic areas without exceeding the normal tissue tolerance $^{32,33}$. Recently, the clinical feasibility of $^{18}$FDG-PET based dose escalation using a uniform dose distribution was demonstrated in a phase I clinical trial on head and neck cancer patients $^6$. With IMRT and simultaneous integrated boost the dose was escalated to 72.5 Gy and 77.5 Gy achieving high local control rates at 1 year of follow-up.

During the initial four weeks of the current planning study, the radiation dose was escalated to 71 Gy in fractions of 2.3 Gy delivered to the highly proliferative subvolumes GTV$_{80\%1}$ and GTV$_{80\%2}$, resulting in a total dose of 74 Gy in the overlapping volume.

In contrast to $^{18}$FDG-PET, which provides a measure of the total viable tumor cell density, $^{18}$FLT-PET identifies the proliferating cell compartment within the gross tumor volume. Although the number of tumor cells is greatly reduced during cytotoxic treatment, cells that survive are triggered to repopulate more effectively during the intervals between treatments, and this process of repopulation is an important cause of treatment failure $^{10,11,35,36}$. Randomized trials have convincingly shown that accelerated radiotherapy, i.e., delivery of the radiation dose in shorter time, can counteract accelerated repopulation and improve the tumor control probability $^{37,38}$. Accelerated radiotherapy is now considered the standard for head and neck cancer. Delivering a higher dose to the most actively proliferating parts of the tumor very early during the treatment course might have an additive effect and could further reduce the potential of the tumor to recover through accelerated proliferation and repopulation. For elderly patients and patients in less good general condition, dose escalation to the highly proliferative subvolumes might be an alternative to accelerated radiotherapy. Accelerated schedules are accompanied by increased toxicity, in particular early mucosal reactions $^{39}$. Dose escalation to a relatively small subvolume using IMRT can be accomplished with only very limited additional burden to the surrounding normal tissues and thus might be better tolerated by these patients. A clinical study will be initiated to further explore the feasibility and effectiveness of this approach.
8.5 Conclusions

\(^{18}\text{FLT}\) is a promising PET tracer for imaging tumor cell proliferation in head and neck carcinomas during treatment. In this study on oropharyngeal tumors, it was shown that \(^{18}\text{FLT}-\text{PET}\) signal changes precede volumetric tumor response assessed by CT or PET and that the tracer is therefore suitable for early response assessment. Furthermore, \(^{18}\text{FLT}-\text{PET}\) can define tumor subvolumes with high proliferative activity, and escalation of radiation dose within these regions is technically feasible. Finally, it must be concluded that at present, adaptive radiotherapy on the basis of \(^{18}\text{FLT}-\text{PET}\) volumetric changes is not possible with the commonly available segmentation tools.

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8.6 References

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Chapter 9

General discussion and future perspectives
Chapter 9

PET may serve many purposes in radiation therapy planning, patient selection for tailored treatment and early tumor response evaluation. In the following paragraphs, the value of PET for characterization of head and neck tumors is discussed and future perspectives leading to individualized radiation therapy are highlighted.

9.1 Tumor characterization

Tumor cell hypoxia, accelerated tumor cell repopulation and intrinsic radioresistance are the three major resistance mechanisms adversely influencing therapeutic outcome in head and neck tumors. Treatment modifications counteracting these tumor features are available at the cost of increased side-effects. Hence, non-invasive tumor characterization prior to and during treatment is desirable aimed at improving treatment outcome, whilst reducing side-effects for patients not profiting.

In this thesis, different methods for imaging CA-IX were compared (chapter 3), and $^{18}$F-fluoromisonidazole ($^{18}$FMISO) and 3'-deoxy-3'-$^{18}$F-fluorothymidine ($^{18}$FLT) were validated in human xenograft tumor models and in primary tumor resection specimen obtained from head and neck cancer patients (chapters 4-7). It was demonstrated that results largely depend on the characteristics of the tracer used, on the quantification methods and on the resolution of the imaging techniques. These findings imply that all potential PET tracers for tumor characterization as well as quantification methods need to be validated against histopathology. Through this process, the strengths and weaknesses of these tracers can be identified as well as potential pitfalls before their introduction into clinical studies.

After the initial, somewhat disappointing results on $^{111}$In-G250, it was decided to temporarily discontinue the development and validation of a radioactive tracer directed against CA-IX, until a high-resolution phosphor imaging device and an animal PET became available. Meanwhile, pre-clinical pilot experiments validating a $^{89}$Zr-F(ab)$_2$-cG250 fragment directed against CA-IX have successfully been performed using small animal PET, autoradiography and CA-IX immunohistochemistry. $^{89}$Zr-labeled cG250-F(ab')$_2$ micro-PET imaging showed rapid accumulation in a head and neck xenograft model, with good correlation to CA-IX expression on a microregional level (publication submitted). Furthermore, a phase I clinical trial validating $^{124}$I-cG250 in head and neck cancer patients was anticipated. However, new European legislation has made the initiation of clinical studies involving new PET tracers very cumbersome.

Based on the encouraging experience in the pre-clinical studies, $^{18}$FMISO-PET is currently being validated against pimonidazole immunohistochemistry in advanced primary head and neck tumor resection specimen at our department (NCT00159978).
There are several possibilities to further improve tumor characterization, e.g., by introducing new PET tracers, optimization of functional CT- and MR applications, or by developing new techniques suitable for multi-modality imaging.

(1) Several groups have been developing other PET tracers potentially suitable for characterization of primary head and neck tumors. Examples are: 1-(2'-deoxy-2'-18F-fluoroarabinofuranosyl)cytosine (18FAC) for imaging nucleoside metabolism, O-2-18F-fluoroethyl-L-tyrosine (18FET) for visualization of protein synthesis, and 1-11C-acetate for imaging non-18FDG avid malignancies 6-8. Before these tracers can be used in the clinic they must be tested in preclinical tumor models and validated against established methods.

Furthermore, the introduction of positron emitting radio-labeled monoclonal antibodies, peptides and other receptor-targeting compounds has broadened the horizon for studying tumor features and individualizing treatment. The epidermal growth factor receptor (EGFR) controls numerous cellular functions, including tumor cell proliferation, apoptosis and angiogenesis. Since the publication of the landmark phase III trial on cetuximab, a chimeric monoclonal antibody directed against EGFR, an increasing number of head and neck cancer patients are undergoing combined treatment with radiotherapy and this drug 9. Additionally, EGFR expression in tumor biopsies was shown to be a predictive factor in hyperfractionated accelerated radiotherapy 10. Hence, it is of great interest to non-invasively image the EGFR expression profile. Radionuclide labeled monoclonal antibodies directed against EGFR have been validated in tumor cell lines and xenograft tumor models including squamous cell carcinomas of the head and neck 11-13. Appropriate sized clinical trials on head and neck cancer patients are necessary to assess the prognostic and predictive value of non-invasive imaging of the EGFR status.

(2) The combination of functional information from PET with the high-resolution anatomical information from CT or MRI can improve clinical decision-making and therapy planning 14,15. Besides, the addition of high-resolution functional MRI- (dynamic contrast enhancement MRI or BOLD-MRI) and CT-studies combined with direct metabolic information gathered by MR spectroscopy and PET may enhance the functional tumor information and thus improve tumor characterization 16,17.

(3) Interesting developments regarding tumor characterization using gold nanoparticles are ongoing 18. Human oral cancer cells overexpressing EGFR at the cell membrane were found to align gold nanorods conjugated to anti-EGFR antibodies at the cancer cell surface 19. These nanorods gave distinct enhanced spectroscopic scattering spectra that may potentially serve as molecular signature unique for cancer. Additionally, these nanoparticles may be used for tumor characterization and possibly...
9.2 Identification of resistant tumor subvolumes

PET may identify radio-resistant tumor subvolumes requiring higher doses of irradiation. Theoretical planning studies have proven the feasibility of this approach for ¹⁸FDG-PET and hypoxic PET tracers 22-27. A recent clinical phase I trial on head and neck cancer patients applying dose escalation to ¹⁸FDG avid subvolumes reported favorable local control rates 28. Results from clinical trials on ¹⁸FMISO and ¹⁸F-fluoroazomycin arabinoside (¹⁸FAZA) guided dose escalation are awaited.

In the meantime, the concepts applied for dose intensification are subject to intense debate. There are concerns about the resolution of hypoxic PET imaging, about its possibility to detect varying levels of hypoxia during the course of treatment, about the dose level required for eliminating radio-resistant tumor cells, and about its prognostic and predictive value.

In this thesis, we contributed to the first and second question regarding the resolution and the changes in oxygenation status (chapter 4 and 5). It was shown that ¹⁸FMISO identified hypoxic areas within the xenograft tumor lines and also the changes in the level of hypoxia. However, these findings largely depended on the underlying microarchitecture of the xenograft tumor line studied. PET imaging has a by far inferior resolution compared to autoradiography. Even though, ¹⁸FMISO readings form the basis for dose escalation studies either by applying uniform doses or by dose-painting by numbers (the higher the ¹⁸FMISO uptake in a voxel, the higher the locally required radiation dose). However, as the tumor microarchitecture influences the ¹⁸FMISO PET readings, caution should be taken when studying relatively small tumor subvolumes.

Furthermore, in chapter 8, the feasibility of escalating radiation dose to ¹⁸FLT-PET derived tumor subvolumes representative of a high density of proliferating tumor cells was successfully addressed. Using IMRT and a SIB technique, the total dose to these subvolumes was escalated to 74 Gy. Very early during the treatment course, delivery of a higher radiation dose to the most actively proliferating parts of the tumor might have an additive effect. It could reduce the proliferative compartment of the tumor, thereby reducing the capacity of accelerated proliferation and repopulation with progression of treatment. For elderly patients and patients with substantial co-morbidity, dose escalation to the highly proliferative subvolumes might be an attractive alternative to accelerated radiotherapy. A clinical study will be initiated to further explore the feasibility and effectiveness of this approach.
As discussed in paragraph 9.1, new tracers and imaging techniques are currently being developed that may further serve the identification of radio-resistant tumor subvolumes. Additionally, developments in the field of autoradiography and PET imaging may improve validation and delineation accuracy. The spatial resolution for human PET-scanners is in the order of 5-7 mm, compared to 1-3 mm for small animal scanners. New developments regarding the size of the detector crystal, the coincidence-timing window and signal processing have achieved a resolution of 2 mm for the human application (product information Siemens). Consequently, the characterization of tumor subvolumes becomes feasible, and their delineation for radiotherapy planning purposes more accurate.

Before the concept of escalating the dose to tumor subvolumes can be introduced in routine clinical practice, it is of utmost importance to balance the benefit in terms of local control and disease-free survival against the additional morbidity in clinical trials. Especially in elderly or multi-morbid patients unfit for treatment modifications (i.e., carbogen and nicotinamide counteracting hypoxia or accelerated radiotherapy compensating for accelerated tumor cell repopulation) dose escalation to small volumes may be an elegant alternative.

9.3 Early treatment response monitoring and adaptive radiotherapy planning

Treatment modifications counteracting tumor cell hypoxia and accelerated repopulation have been successfully developed 2-5. However, they increase the severity and duration of acute side-effects 29,30. Hence, it is desirable to non-invasively characterize the tumor prior to therapy for individualized treatment selection and to monitor response early during treatment facilitating adaptations of the treatment.

In chapter 8 of this thesis, the value of $^{18}$FLT-PET for early treatment response monitoring during radiotherapy was addressed in oropharyngeal cancer patients. Overall, $^{18}$FLT-PET signal intensities as measured by the standardized uptake value (SUV) already decreased more than two-fold in the second week, and even further in the fourth week of treatment. On an individual basis, different response patterns became apparent. Some patients only showed a modest reduction in SUV, whilst others even responded with an increase. It is to be awaited, whether these inter-individual discrepancies will have discriminative power for tumor response assessment.

The cohort discussed was part of a larger clinical trial on 52 patients with advanced stage head and neck cancer undergoing repetitive $^{18}$FLT-PET-CT imaging during the course of radiotherapy or chemoradiation (NCT00163176). This study was closed September 2008 and the results with respect to the prognostic power of $^{18}$FLT-PET are expected to be published in the autumn of 2010.
As mentioned in paragraph 9.1, EGFR activation influences the tumor’s proliferative activity. Therefore, our group is currently assessing early treatment-induced changes of the $^{18}$FLT-PET signal in head and neck patients undergoing radiotherapy combined with cetuximab. With this study, we aim to understand more about the tumor response to cetuximab alone and about the additional value of combined treatment. Furthermore, we investigate whether $^{18}$FLT-PET can distinguish patients benefiting from the addition of cetuximab from those only experiencing its side-effects and thus guide tailored treatment.

Additionally, the feasibility of adaptive image-guided radiotherapy using $^{18}$FLT-PET was addressed in chapter 8. Earlier, in a proof of principle study, Geets et al. assessed the concept of adaptive image-guided radiotherapy in head and neck cancer patients\(^\text{31}\). Radiation treatment planning based on repetitive $^{18}$FDG-PET scanning and volume adaptation progressively reduced the irradiated volumes compared to traditional CT-based treatment prior to irradiation.

In our study (chapter 8), a significant reduction in CT derived gross tumor volumes was only observed in the fourth week of treatment. $^{18}$FLT-uptake decreased much more rapidly, but a limitation was that, with progression of treatment, it was increasingly difficult to segment tracer uptake in the tumor from the background. This was caused by the decreased $^{18}$FLT signal and increased tracer uptake in the tonsillar region, most likely caused by proliferating inflammatory cells. As a result, accurate tumor delineation and adaptation of the treatment plan during irradiation was not possible. The value of $^{18}$FLT-PET for adaptive radiotherapy planning in other head and neck subsites is yet to be assessed.

There are new developments regarding tumor segmentation that may contribute to adaptive radiotherapy planning. Our group has been involved in the development and validation of an iterative background-subtracted relative-threshold level (RTL) method\(^\text{32}\). Geets et al. have validated a gradient-based segmentation tool based on watershed transform and hierarchical cluster analysis\(^\text{33}\). These segmentation methods will shortly be tested on the patient cohort. However, it remains to be seen whether these two new segmentation tools can solve the encountered difficulties.

The tumor microenvironment is a highly dynamic system with continuous cell turnover occurring both with or without treatment\(^\text{34,35}\). In order to accurately assess these changes, it is compulsory to repetitively image the tumor during the course of therapy. This may be facilitated by the development of specific PET tracers with a shorter half-life compared to $^{18}$F. Using compounds labeled with $^{11}$C (half-life 21 min) and $^{15}$O water (2 min), the acquisition of two PET scans within a short time span becomes feasible, e.g., for imaging multiple tumor characteristics at one day or for instant tumor response.
evaluation after treatment modification (e.g., after carbogen breathing). Besides, double tracer techniques combining a shorter and a longer half-life radiopharmaceutical become attainable.

9.4 Final remark

Since the discovery of x-rays and natural radioactivity just over one hundred years ago, the three disciplines Radiation Oncology, Nuclear Medicine and Radiology have gradually evolved. In the field of oncology, these three specialties are moving closer together, e.g., by combining anatomical and functional imaging devices for tumor characterization and treatment planning. The increasing role of PET for tailor-made radiotherapy is best highlighted by the emergence of PET-CT scanners into Radiotherapy Departments.

9.5 References


Chapter 10

Summary and conclusions
Several aspects of visualizing and quantifying the biological tumor characteristics tumor cell hypoxia and proliferation by means of PET were discussed in this thesis. In chapter 2, an introductory overview regarding the role of $^{18}$FDG-PET for radiotherapy planning purposes was given and more specific tracers were put forward.

10.1 Tumor cell hypoxia

The value of $^{111}$In-G250, a radio-labeled antibody directed against the hypoxia-related marker CA-IX, was assessed in chapter 3. $^{111}$In-G250 biodistribution was compared with pimonidazole and CA-IX immunohistochemistry in a panel of human head and neck carcinoma xenograft lines. It was concluded that different methods of CA-IX quantification resulted in different findings. Furthermore, the immunohistochemical staining pattern of CA-IX relative to pimonidazole differed between tumor lines of similar origin. Therefore, the role of CA-IX as a marker of tumor hypoxia was disputed.

In chapter 4 and 5, $^{18}$FMISO was validated in human xenograft tumor lines. First, $^{18}$FMISO autoradiography was compared with pimonidazole immunohistochemistry using pixel-by-pixel analysis, mean signal intensities and hypoxic fraction (chapter 4). Overall, a weak but significant correlation between hypoxic fractions based on pimonidazole binding and the mean $^{18}$FMISO signal intensity was observed. However, for tumors with a ribbon-like microregional distribution pattern of hypoxia the correlation was much stronger. This indicates that $^{18}$FMISO accumulation depends on the underlying microregional distribution of hypoxia.

In a subsequent study including three xenograft tumor lines (chapter 5), the level of hypoxia was altered by clamping of the tumor and by carbogen breathing. Again, a significant, but tumor line dependent correlation between the hypoxic fractions and the mean signal intensities of pimonidazole and $^{18}$FMISO was observed. The hypoxic fraction based on pimonidazole staining significantly increased and decreased in all xenograft tumors. However, an increased $^{18}$FMISO signal after tumor clamping was only observed in two of the three lines, and there was no reduction of the $^{18}$FMISO signal after carbogen breathing.

It was concluded that $^{18}$FMISO can be used for the detection of tumor cell hypoxia and for monitoring treatment-induced changes in the level of hypoxia. However, caution must be taken when studying relatively small tumor subvolumes as $^{18}$FMISO accumulation depends on the tumor microarchitecture.

10.2 Tumor cell proliferation

In chapter 6 and 7, $^{18}$FLT-PET was validated for the characterization of primary oral cavity tumors and for the detection of cervical lymph node metastases. Seventeen patients with oral cavity tumors underwent an $^{18}$FLT-PET scan prior to tumor resection,
Summary and conclusions

and iododeoxyuridine was intravenously administered shortly before the surgery (chapter 6). There was a significant correlation between the $^{18}$FLT-PET standardized uptake values and iododeoxyuridine optical densities. Unexpectedly, expression of TK-1, the principal enzyme in $^{18}$FLT phosphorylation, did not correlate with $^{18}$FLT-uptake. This was probably the result of differences in biomarker characteristics, resolution and quantification methods.

In ten patients with squamous cell carcinoma of the head and neck undergoing a lymph node dissection, the value of $^{18}$FLT-PET for identifying metastatic deposits was assessed (chapter 7). In PET positive lymph nodes, abundant Ki-67 and iododeoxyuridine staining of B-lymphocytes was found in germinal centers. Therefore, it was concluded that due to the high rate of false positive findings, $^{18}$FLT-PET is not suitable for assessment of the pre-therapeutic lymph node status.

In chapter 8, various aspects of repetitive $^{18}$FLT-PET-CT imaging were addressed in ten advanced stage oropharyngeal cancer patients. $^{18}$FLT-PET-CT scans were acquired before the start of (chemo)radiation and in the second and fourth week of treatment. Changes in $^{18}$FLT-PET standardized uptake values were found to precede volumetric tumor changes on CT. Tumor subvolumes with a high density of proliferating cells were successfully identified on $^{18}$FLT-PET, and the technical feasibility of dose escalation to these regions was shown. A limitation is that, with progression of treatment, it was increasingly difficult to segment tracer uptake in the tumor from the background due to the decrease of the $^{18}$FLT signal and increased uptake in nearby tonsillar tissue.

Chapter 9 provides a general discussion of the presented findings and highlights future perspectives.

10.3 Conclusions

Several PET tracers to image biological tumor characteristics reflecting radiation resistance mechanisms are available and offer potential for tailored radiation therapy. In this thesis, validation of imaging with tracers visualizing tumor cell hypoxia ($^{111}$In-G250 and $^{18}$FMISO) and proliferation ($^{18}$FLT) was performed in human xenograft tumors and in primary head and neck resection specimen. Furthermore, the value of $^{18}$FLT-PET for early treatment response measurement, adaptive image-guided radiotherapy planning and focal dose escalation was assessed.
Chapter 11

Samenvatting en conclusies
11.1 Hoofd- halskanker

Kanker in het hoofd- halsgebied (vaak een zogenaamd plaveiselcelcarcinoom afkomstig van het slijmvlies) wordt jaarlijks bij ongeveer 2400 in Nederland wonende mensen vastgesteld. Veelal hebben patiënten in het verleden gerookt of alcohol geconsumeerd, maar er is ook in toenemende mate onderbouwing dat een virus, het humaan papillomavirus (HPV), vooral bij jonge mensen kanker in het gebied van de amandelen, het zachte gehemelte of de tongbasis kan veroorzaken. De klachten van patiënten zijn meestal vaag en daarom presenteren zij zich veelal met een gevorderd stadium van de ziekte. Zij hebben dan grote primaire tumoren en uitzaaïngen (metastasen) naar lymfklieren in de hals. Hoofd- halskanker is in eerste instantie een locale ziekte die een locale behandeling vereist: chirurgische verwijdering van de primaire tumor en de lymfkliermetastasen, bestraling (radiotherapie) van de tumor en (potentieel) aangedane lymfklieren, chemotherapie ter verbetering van het effect van de bestraling, of een combinatie van deze behandelmoelijkheden. Radiotherapie is door het orgaanbehoud in de meeste gevallen de belangrijkste pijler van de behandeling. In de jaren 90 van de vorige eeuw heeft de radiotherapie een belangrijke ontwikkeling doorgemaakt met de introductie van “intensiteitgemoduleerde radiotherapie” (IMRT). Met deze techniek kan de tumor tot een hoge bestralingsdosis behandeld worden met gelijktijdige sparing van omliggend gezond weefsel. Echter, om deze methode toe te kunnen passen is het van groot belang de exacte tumoruitbreiding te kennen.

11.2 Afbeelding van hoofd- halstumoren

Het in kaart brengen van een tumor in het hoofd- halsgebied begint met een grondig lichamelijk onderzoek: inspectie van mond- en keelholte (indien nodig tevens onder algehele narcose) en aftasten van de hals op zoek naar vergrote lymfklieren. Daarnaast worden beeldvormende technieken toegepast om de tumor en halslymfklieren in kaart te brengen (anatomische beeldvorming): computertomografie (CT) en magnetische resonantie (MR). CT kan aantasting van kraakbeen of bot aantonen (voornamelijk voor kanker van het strottenhoofd), MR geeft een hoog wekedelen contrast teneinde onderscheid te kunnen maken tussen bijvoorbeeld diepe tongspieren en tumor (geschikt voor tumoren van mondholte, tong, amandelen, zacht gehemelte en tongbasis). Beide beeldvormende modaliteiten kunnen ook gebruikt worden om de reactie van de tumor op behandeling in kaart te brengen. Echter, zij hebben nadelen: (1) het kan moeilijk zijn onderscheid te maken tussen tumor en omliggend normaal weefsel, (2) verkleining van het tumorvolume is vaak pas in een late fase van de bestraling meetbaar, en (3) na de behandeling kan de anatomie veranderd zijn, enerzijds als reactie op nog aanwezige kwaadaardige ziekte of anderzijds door volledig verdwijnen van de tumor. Daarom zijn recent verschillende “functionele
beeldvormende technieken” geïntroduceerd, die informatie geven over biologische eigenschappen van het afgebeelde weefsels.

Positron emissie tomografie (PET) is een relatief nieuwe functionele beeldvormende techniek. Hiermee kan van buiten af (niet-invasief) een radioactief gemarkeerde stof afgebeeld en gekwantificeerd worden. Met de meest gebruikte radioactieve stof, 18F-fluorodeoxyglucose (18FDG; radioactief fluor gekoppeld aan een variant van suiker), kan het energieverbruik van verschillende weefsvellen in kaart worden gebracht. Betreffende de bestralingsplanning zou 18FDG-PET belangrijke aanvullende informatie over de tumoruitbreiding kunnen geven. Vaak gaan hoofd- halstumoren echter gepaard met lokale ontstekingsreacties rond de tumor en met opgezette “reactieve” lymfklieren. Omdat 18FDG ook in gebieden van ontstekingen wordt opgenomen, is de waarde van deze markerstof voor deze specifieke tumoren dus slechts beperkt. Naast het afbeelden en afgrenzen van tumoren kan PET ook worden gebruikt voor het karakteriseren daarvan. Dit is de doorlopende vraagstelling van dit proefschrift.

11.3 Ongunstige tumoreigenschappen voor radiotherapie

Er zijn drie belangrijke tumoreigenschappen die de uitkomst van een bestralingsbehandeling nadelig beïnvloeden: zuurstofgebrek (hypoxie), versnelde deling (proliferatie) van tumorcellen tijdens de bestraling (geaccelereerde repopulatie) en de tumoreigen gevoeligheid voor bestraling die per tumor verschilt (intrinsieke radioresistentie). “Acute hypoxie” ontstaat door het willekeurig en kortdurend sluiten van slecht gevormde tumorbloedvaten die de zuurstoftoevoer en -spanning doen variëren. “Chronische hypoxie” is het gevolg van een te grote afstand van de tumorcel naar het dichtstbijzijnde bloedvat, met als gevolg een constant tekort aan zuurstof en voedingsstoffen. Geaccelereerde repopulatie is een vaak optredende reactie van hoofd- halstumoren om het celverlies, dat tijdens de bestraling ontstaat, tegen te gaan. Hoe langer de totale bestralingsbehandeling duurt, hoe slechter de uiteindelijke behandeling uitkomst. De tumoreigen gevoeligheid voor bestraling wordt onder andere bepaald door factoren zoals de celegeen capaciteit om bestralingsschade aan het DNA te herstellen.

Er zijn verschillende behandelingsmogelijkheden om deze ongunstige tumoreigenschappen tegen te gaan. Door verkorten van de totale behandelduur wordt de versnelde deling van tumorcellen tegengegaan. Dit wordt in de praktijk gerealiseerd door twee maal per dag te bestralen of door ook gedurende de weekenden te behandelen. Ook hypoxie kan op verschillende manieren worden tegengegaan: er zijn geneesmiddelen, zogenaamde “hypoxische sensitisizers”, die zuurstofarme cellen gevoeliger maken voor bestraling, en men kan patiënten tijdens de behandeling extra zuurstof laten inademen. Recent is een door het UMC St Radboud gecoördineerde
landelijke klinische studie bij patiënten met strottenhoofdkanker afgerond. Patiënten werden versneld bestraald, ademden carbogen (98% zuurstof en 2% koolstofdioxide) en namen een geneesmiddel voor verwijding van de bloedvaten in om acute hypoxie tegen te gaan (Accelerated Radiotherapy with CarbOgen breathing and Nicotinamide, ARCON). Uitkomsten van deze studie worden in 2010 verwacht.

11.4 Invasieve methoden voor tumorkarakterisering

Zuurstofgebrek kan gemeten worden met behulp van een optische sensor, die in de hoofd- halstumor of lymfkliermetastase wordt ingebracht. Daarnaast kan men zowel hypoxie als ook celdelingactiviteit van een tumor op speciaal gekleurde weefselcoupes microscopisch aantonen (immunohistochemie). Beide methoden hebben echter beperkingen: zij zijn invasief, beelden maar een gedeelte van de tumor af, en kunnen zonder narcose alleen toegepast worden bij makkelijk toegankelijke tumoren. Hierdoor is het herhaaldelijk analyseren van tumorkarakteristieken tijdens de behandeling slechts mogelijk bij een deel van de tumoren. Niet-invasieve methoden, zoals PET, kunnen een tumor in zijn geheel (macroscopisch) voor en ook tijdens de bestraling in kaart brengen.

11.5 Tumorkarakterisering met PET

In hoofdstuk 2 van dit proefschrift werden onder andere de verschillende PET markerstoffen besproken, die specifiek zuurstofgebrek en celdelingactiviteit in tumoren kunnen afbeelden (paragraaf 2.3).

11.5.1 Zuurstofgebrek

Verschillende methoden om carbonische anhydrase IX (CA-IX), een lichaamseigen aan hypoxie gerelateerd enzym aan te tonen, werden in hoofdstuk 3 vergeleken. Hiervoor werden op de flank van naakte muizen biopten van menselijke tumoren geïmplanteerd en tot groei gebracht (xenografts). Na injectie van 111In-G250, een radioactief antilichaam tegen CA-IX, werden weefselcoupes van deze tumoren met autoradiografie (afbeelding van radioactiviteit op een fosforplaat) en immunohistochemie afgebeeld en de 111In-G250 opname in de weefsels ("biodistributie") kwantitatief bepaald. De belangrijkste conclusies van dit onderzoek waren dat CA-IX niet zonder meer als een vervanger van de als standaard gebruikte hypoxie marker pimonidazole beschouwd mag worden en dat verschillende methoden om CA-IX te meten niet direct vergelijkbaar zijn.

18F-fluoromisonidazole (18FMISO) is een markerstof voor PET die in cellen met zuurstof gebrek wordt opgenomen. Met behulp van 18FMISO kunnen hypoxische delen van de tumor worden geïdentificeerd. Voorts is het theoretisch mogelijk, deze delen middels
speciale IMRT technieken te bestralen. Echter, er zijn nog een aantal onduidelijkheden betreffende de waarde van $^{18}$FMISO-PET. In dit proefschrift werd $^{18}$FMISO in weefselcoupes met behulp van autoradiografie opgespoord en de verdeling hiervan vergeleken met middels immunohistochemie aangetoonde hypoxie (gebruikte markerstof pimonidazole).

In hoofdstuk 4 werden weefselcoupes van tien verschillende menselijke hoofd- halstumor xenografts met behulp van beide beeldvormende technieken in kaart gebracht. Vastgesteld werd dat de verdeling van $^{18}$FMISO afhankelijk is van het hypoxie patroon op microregionaal niveau. Verder werd slechts in vijf tumoren, allen met dezelfde microscopische opbouw, een overeenkomst tussen de signaalintensiteiten van beide beeldvormende technieken gevonden. De conclusie van dit onderzoek is dat weergave van hypoxie door $^{18}$FMISO-PET afhankelijk is van het onderliggend microregionaal hypoxie patroon. Hiermee dient rekening te worden gehouden indien men $^{18}$FMISO-PET wil gebruiken voor identificatie van hypoxische tumorvolumina ten behoeve van IMRT.

Daarnaast onderzochten wij in hoofdstuk 5 in drie verschillende xenograft tumorlijnen of veranderingen in de mate van hypoxie ook middels $^{18}$FMISO autoradiografie aangetoond konden worden. Pimonidazole immunohistochemie was opnieuw de standaard waarmee vergeleken werd. In één groep werd de mate van hypoxie verhoogd door mechanische onderbinding van de bloedtoevoer (clamping); in een andere groep werd de hypoxie verminderd door inademen van carbogeen. De bevindingen waren dat $^{18}$FMISO de toename van hypoxie in twee van de drie onderzochte xenografts kon aantonen, maar dat de afname niet detecteerbaar was. Verder werd voor twee van de drie tumorlijnen een significante samenhang tussen de hypoxische fractie en de signaalintensiteiten van pimonidazole en $^{18}$FMISO aangetoond.

### 11.5.2 Tumor cel proliferatie en repopulatie

3'-deoxy-3'-$^{18}$F-fluorothymidine ($^{18}$FLT) is een PET markerstof die delende cellen opsporrt. In theorie is dit juist voor tumoren in het hoofd- halsgebied een bij uitstek geschikte marker. Voordat deze in de klinische praktijk geïntroduceerd kan worden is echter het vaststellen in welke mate $^{18}$FLT op betrouwbare wijze proliferatie weergeeft (validatie) noodzakelijk.

Hiervoor werd in hoofdstuk 6 bij zeventien hoofd- halspatiënten een $^{18}$FLT-PET scan vervaardigd alvorens zij een chirurgische verwijdering van de tumor zouden ondergaan. De informatie van het PET signaal werd vergeleken met microscopische bevindingen in weefselcoupes van de verwijderde tumor (gebruikte markerstof iododeoxyuridine: standaard proliferatiemarker bij immunohistochemie). Er was slechts een zwakke maar wel statistisch significante samenhang tussen het $^{18}$FLT-PET signaal en de signaalintensiteit van iododeoxyuridine. Deze bevindingen waren
waarschijnlijk het gevolg van verschillen in beeldresolutie en van specifieke kenmerken van beide merkstoffen.

De waarde van $^{18}$FLT-PET voor het opsporen van halsklirmetastasen werd in hoofdstuk 7 onderzocht. Bij tien hoofd- halspatiënten die een verwijdering van de halsklieren ondergingen werd eveneens een $^{18}$FLT-PET scan gemaakt. Vervolgens werden de PET bevindingen vergeleken met de celdelingactiviteit in weefselcoupes van verwijderde lymfklieren. Verrassend genoeg werd het $^{18}$FLT-PET signaal foutief beïnvloed door delende ontstekingscellen in de halslymfklieren. Derhalve werd geconcludeerd dat $^{18}$FLT-PET (evenals $^{18}$FDG-PET) bij deze tumorsoort geen toegevoegde waarde heeft voor het opsporen van uitzaaiingen naar de lymfklieren.

In hoofdstuk 8 werden bij tien patiënten met hoofd- halskanker de veranderingen van het $^{18}$FLT-PET-CT volume en signaal bestudeerd voor en tijdens bestraling, met of zonder chemotherapie ($2^e$ en $4^e$ week). De veranderingen in het $^{18}$FLT-PET signaal waren al in de tweede week van de behandeling (na minimaal 5 bestralingen) duidelijk meetbaar en liepen vooruit op volumeveranderingen op CT. Verder kon een tumorgebied gedefinieerd worden, waarbinnen de celdelingactiviteit zeer hoog was. Een theoretische planningstudie slaagde erin om dit tumorvolume met een extra hoge dosis te bestralen zonder omliggend gezond weefsel te schaden.

### 11.6 Conclusies

Radiotherapie is bij patiënten met hoofd- halskanker veelal de behandeling van keuze, al of niet in combinatie met chirurgie of chemotherapie. Naast lichamelijk onderzoek en anatomische beeldvorming heeft functionele beeldvorming met PET tot nu toe geen toegevoegde waarde voor het vinden van de tumor of lymfklieruitzaaiingen. Het gebruik van PET voor het definiëren van de tumoruitbreiding ten behoeve van bestralingsplanning is nog onderwerp van onderzoek. Sommige PET markerstoffen kunnen biologische eigenschappen van de tumor voor en tijdens de behandeling gedetailleerd in kaart brengen op basis waarvan mogelijk de behandeling verder geïndividualiseerd kan worden. Daarnaast komen er aanwijzingen dat op basis van deze biologische eigenschappen tumorgebieden aangetoond kunnen worden waarvoor het wellicht zinvol kan zijn een hogere bestralingsdosis toe te dienen om zo de genezingskans te vergroten. Voordat deze PET markerstoffen echter klinisch toepast kunnen worden, moeten de bevindingen gevalideerd worden. De ontwikkeling van nieuwe markerstoffen en de technische vooruitgang van PET zullen de nauwkeurigheid en individualisatie van radiotherapie waarschijnlijk doen toenemen met uiteindelijk als resultaat een betere controle van de tumor en een beperking van bijwerkingen.
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Hora est.
1. Curriculum vitae


In 2008 promoveerde zij (magna cum laude) aan de Medizinische Hochschule Hannover te Hannover (Duitsland) op het proefschrift getiteld "Interleukin polymorphisms in Helicobacter pylori associated gastric cancer" (promotores: prof. dr. S. Suerbaum, afdeling Microbiologie, Medizinische Hochschule Hannover, en prof. dr. E.M. El-Omar, afdeling Gastroenterologie, Aberdeen University).

2. Publicaties


**Troost EGC**, Bussink J, Hoffmann AL, Boerman OC, Oyen WJG, Kaanders JHAM. “$^{18}$FLT-PET-CT for early response monitoring and dose escalation in oropharyngeal tumors”. Accepted for publication in J Nucl Med.


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