Chapter 8

In this thesis, two challenges in the diagnosis and treatment of head and neck squamous cell carcinoma (HNSCC) are described: staging the clinically negative (cN0) neck with the sentinel node (SN) procedure, and improving survival using targeted therapies. For both challenges, the use of molecular imaging methods (e.g. PET/CT or NIR fluorescence imaging) may play a prominent role in improving current clinical practice. Molecular imaging as referred to in this thesis differs from traditional imaging in the fact that exogenously administered tracers are used to enable imaging of particular (targeting) ligands, targets or pathways.

Improving diagnosis of the clinically negative neck using the sentinel node procedure

One of the challenges in the diagnosis of HNSCC is the correct staging of the neck in case of the presence of very small (occult) lymph node metastases. In the introduction of this thesis (chapter 1), it is concluded that palpation and conventional staging techniques like ultrasound (US), computerized tomography (CT), magnetic resonance imaging (MRI), $^{18}$F-fluorodeoxyglucose-positron emission tomography (FDG-PET), and US-guided fine needle aspiration cytology, are not reliably able to detect these occult lymph node metastasis. This lack of diagnostic accuracy becomes especially important in the diagnosis of T1-T2 oral cavity cancers, in which the risk of occult metastasis is about 30%. In these patients, the primary tumour often can be resected transorally and in that situation the dilemma exists how to treat the neck: elective neck dissection with surgical overtreatment of about 70% of patients or following a 'watchful waiting' policy with the risk of undertreatment in case of metastastic disease.

The SN procedure has been introduced as a diagnostic staging procedure in early stage oral cancer patients and aims to select those patients who really benefit from a neck dissection. The SN is defined as the first lymph node that receives lymphatic drainage from the tumour area and therefore, the SN should always contain metastatic disease in case of lymphogenic spread. Often, there are multiple SNs. The procedure consists of three steps: (1) preoperative identification of the SN based on lymphoscintigraphic imaging; (2) surgical removal of the SN with the help of a handheld gamma probe and blue dye injection; and (3) thorough histopathological examination of the SN using step-serial sectioning and immunohistochemical stainings.

Despite a good overall performance, there is still room for improvements of this procedure in oral cavity cancers. This concerns especially floor of mouth (FOM) tumours, in which (significant) lower sensitivity and negative predictive value have been reported. This is because of the "shine-through" phenomenon, a result of the short distance between SNs and the FOM tumour, in combination with the limited resolution of currently used techniques, i.e. planar lymphoscintigraphy and SPECT/CT using the colloid Nanocoll (in Europe) labelled with the gamma emitter technetium-99m ($^{99m}$Tc, $t_{1/2}=6$ h) as tracer ($^{99m}$Tc-Nanocoll). Since most of the peritumourally injected tracer will remain at the injection site, a large focus will be produced on the lymphoscintigraphy images. Uptake of the tracer in a SN close to the primary tumour may be hidden by the large focus of the injection site and such a SN may not be visualized and thus not be identified.

The limited resolution may also give problems if complex lymphatic drainage is observed with
visualization of multiple foci. The timing of imaging is important with respect to the visualization of lymphatic drainage and uptake in the SN and second echelon lymph nodes. It is known that there is an increased possibility that the tracer will be migrated beyond the SN towards the second echelon lymph nodes if imaging is performed at a late time point following injection of the tracer. As a consequence, it may be unclear whether a focus visualized on lymphoscintigraphy should be considered to be a true SN or a second echelon lymph node. Such a focus may be falsely considered to be SN, resulting in a futile surgical removal of a second echelon lymph node.

In chapter 2, the clinical value of routinely performing additional late lymphoscintigraphic imaging by means of repeated planar lymphoscintigraphy or SPECT/CT is evaluated. In this study, a retrospective analysis of the early (within 30 minutes after injection of $^{99m}$Tc-Nanocoll) and late (2-4 h after injection) lymphoscintigraphic imaging results of 60 early stage oral cancer patients was performed. In 9 of 60 patients, SNs were visualized only during late imaging, whereas in 51 of 60 patients, early imaging demonstrated visualization of a focus considered to be a SN. Routine performed late lymphoscintigraphy revealed additional foci marked as SN in 14 of these 51 patients (27%). This resulted in a more extensive surgical procedure, while these additionally removed SNs were of no clinical relevance, as all SNs identified during early imaging correctly predicted whether the neck was positive or negative for cancer. Furthermore, this study showed that lymphatic drainage appeared to be slower for tumours in the oral cavity other than mobile tongue and FOM tumours, and for paramedian or midline tumours in which bilateral drainage often can be observed. Therefore, in order to minimize the risk of false-negative results, we concluded that additional late lymphoscintigraphic imaging should always be considered for these selected tumours. In the remaining patients, late lymphoscintigraphic imaging should only be performed if there is no focus visible considered to be a SN at early imaging. As a result of this, the extent of the SN biopsy procedure may be reduced, making the procedure as minimal invasive as possible. This study also nicely demonstrated the difficulty of differentiating between a true SN and possible second echelon lymph node on current imaging methods. Another aspect of this study was the assessment of the value of blue dye for facilitating the intraoperative visualization of the SN: blue staining of the SN was visible in just 55% of excised SNs. Thus, in 45% of excised SNs the SN could not be visualized by blue dye during the surgical procedure.

In the complex anatomy of the neck, the lack of visualization of the SN may result in extensive surgical exploration in order to find the SN, resulting in fibrosis postoperatively. The latter may negatively influence a possible staged neck dissection in case of a positive SN. Moreover, detection can especially become difficult if the SN is located close to the primary due to the large amount of radioactivity arising from the primary (e.g. FOM) tumour, i.e. injection site. In order to improve the intraoperative visualization of the SN in the current procedure, a portable freehand SPECT device, providing 3-dimensional navigation towards the SN, has been developed which could be used during the SN biopsy procedure. Chapter 3 describes the first series (n=29) of early stage oral cancer patients in which this device was evaluated. Freehand SPECT was able to visualize 95% of all preoperatively identified foci on lymphoscintigraphy during the surgical procedure. Four preoperatively identified foci could not be identified intraoperatively.
of which 3 of them were located in level Ib of the neck in three patients. Two of the three patients did have a tumour in the FOM. Intraoperative level Ib SN detection rates, although small numbers, were 75% for FOM tumours and 88% for tongue tumours. Although not statistically significant, the results of this study may suggest that even with the use of freehand SPECT difficulties may occur in the intraoperative detection of SNs which are located close to the primary (FOM) tumour. However, the use of freehand SPECT appeared to be an additional help in localising the SN in 38% of cases, as assessed by a subjective questionnaire. Thus, its use may have a role in minimizing the surgical damage and the postoperative development of fibrosis.

As mentioned above, preoperative identification of a SN located very close to the injection site, which is the case for FOM tumours, may be difficult. The large focus of the injection site may hide possible SNs located in the close proximity of the tumour, and therefore the true SN may not be identified preoperatively. In order to improve the preoperative identification of such SNs, an imaging technique is needed that provides higher resolution images, which may be achieved by using PET. To allow for high-resolution PET lymphoscintigraphy, a PET tracer was needed and had to be developed. In chapter 4, the development and preclinical evaluation of a novel PET tracer dedicated to SN detection is described.

Instead of labelling of Nanocoll with $^{99m}$Tc, a protocol was designed for labelling of the positron emitter zirconium-89 ($^{89}$Zr, $t_{1/2}=78.4$ h) to Nanocoll ($^{89}$Zr-Nanocoll). For successful labelling, the Nanocoll first had to be modified with the bifunctional chelate $\beta$-isothiocyanatobenzyl desferrioxamine B (Df-Bz-NCS). Hereafter, coupling of $^{89}$Zr to the modified Nanocoll appeared to be efficient, resulting in a stable product with a radiochemical purity greater than 95%. Particle size measurements using atomic force microscopy demonstrated that this procedure had no influence on the particle size of the Nanocoll particles. These results were similar to the quality control specifications of $^{99m}$Tc-Nanocoll. For preclinical in vivo validation studies, a rabbit lymphogenic metastasis animal model was used. This animal model resembles head and neck cancer and is an excellent model for SN evaluation studies. To get information about tracer distribution, imaging studies were carried out in which PET imaging was compared with conventional imaging. PET imaging using $^{89}$Zr-Nanocoll showed a similar drainage pattern, but at higher resolution. Moreover, PET imaging allowed for 3-dimensional visualization of connecting lymphatic vessels, through which differentiation between SN and second echelon lymph nodes became more reliable. Next to the imaging studies, comparative biodistribution experiments were carried out at different time points after coinjection of $^{99m}$Tc- and $^{89}$Zr-Nanocoll, which showed a similar uptake of both tracers in the SN ($R^2=0.99$), again demonstrating identical tracer kinetics.

Because there were no differences between $^{89}$Zr- and $^{99m}$Tc-Nanocoll with respect to the characteristics and in vivo behaviour, and the production of $^{89}$Zr-Nanocoll could be performed according to current Good Manufacturing Practice (cGMP), a clinical pilot study was initiated. In chapter 5, the results of this study are described. This study was performed in five early stage oral cancer patients, who were scheduled for a SN procedure. $^{89}$Zr-Nanocoll (1-2 MBq) was injected peritumorally, followed by dynamic PET/CT imaging, which was repeated the next day. About one week later, the same patient underwent the routine SN procedure using $^{99m}$Tc-Nanocoll (100 MBq), conventional planar lymphoscintigraphy and
SPECT/CT imaging, followed by SN biopsy the next day. The imaging results of PET/CT and SPECT/CT were compared with respect to SN identification and tracer distribution. A similar drainage pattern was observed for both tracers, whereas PET/CT provided higher resolution images with additional SNs visualized which were located near the (FOM) tumour, and in some cases visualization of connecting lymphatic vessels. The latter resulted in a better differentiation between a true SN and a second echelon lymph node in one case. The results of this pilot study demonstrated the feasibility of PET/CT lymphoscintigraphy using $^{89}$Zr-Nanocoll.

For further improvement of the intraoperative visualization of the SN the use of near-infrared (NIR) fluorescence imaging is a promising technique. NIR fluorescence imaging can be used for detection of superficial NIR fluorescence labelled structures. At the moment, the only NIR fluorescent dye which is clinical allowed for SN detection is indocyanine green (ICG). However, ICG has limited retention time in the SN and its use is therefore limited to a short period of time. In chapter 6, the development, characterization, and preclinical evaluation of a novel NIR fluorescent tracer with optimal characteristics for intraoperative SN detection is described. The fluorescent dye IRDye800CW, one of the best NIR fluorescent dyes available at this moment, was conjugated to Nanocoll (Nanocoll-IRDye800CW). Conjugation (achieved in a cGMP compliant way) resulted in a pure and stable product without affecting the particle size of the Nanocoll particles as assessed by atomic force microscopy. With respect to the fluorescence properties of the tracer the quantum yield was determined and compared to that of ICG mixed with human serum albumin (ICG/HSA. Results of these measurements showed a similar quantum yield for both tracers. In vivo validation of Nanocoll-Irdye800CW, performed in the same rabbit animal model as described above, demonstrated optimal retention time in the SN, up to 24 h after injection, which appeared to be superior compared with ICG/HSA. Noninvasive detection of the SN was possible and NIR fluorescence-guided resection of the SN demonstrated an excellent contrast ratio between the Nanocoll-IRDye800CW labelled lymph node and the surrounding tissue. Therefore, Nanocoll-IRDye800CW may be considered as a promising NIR fluorescent tracer for SN detection.

Although the overall performance of the SN procedure in early stage oral cancer is very good, improvements can be achieved for further optimization of the procedure. With the introduction of the novel PET-tracer $^{89}$Zr-Nanocoll the preoperative detection rate of SNs which are located near the primary tumour may be improved, possibly resulting in a better performance of the procedure in, for example, FOM tumours. For future use of this tracer, technologies have to be developed or evaluated to figure out which one is best suitable for intraoperative detection of $^{89}$Zr-Nanocoll containing lymph nodes. Examples are a handheld photon probe especially dedicated for detection of 511 keV gamma rays, the use of a β-probe which detects the positron ($\beta^+$) particles, the use of NIR fluorescence imaging with e.g. Nanocoll-IRDye800CW as optimal fluorescent tracer, combinations of aforementioned options, or other novel innovating techniques.

An important aspect that has to keep in mind are the associated high costs of performing a PET/CT scan, and the (limited) availability of PET/CT cameras. Therefore, careful patient selection should be a priority: $^{89}$Zr-Nanocoll should only be offered to patients with tumours that have known first
draining lymph nodes near the tumour. On the other hand, $^{89}$Zr-Nanocoll is the only available tracer for SN detection (not using $^{99m}$Tc) with exactly the same in vivo behaviour as $^{99m}$Tc-Nanocoll. Therefore, it seems that $^{89}$Zr-Nanocoll may be considered as a reliable alternative for $^{99m}$Tc-Nanocoll. This may become especially important in case of worldwide shortage of $^{99m}$Tc, which is reported to become a potential serious concern. Furthermore, the use of $^{89}$Zr-Nanocoll and Nanocoll-IRDye800CW is not only limited to oral cancer patients, but can be used for all tumour types in which the SN procedure is applied.

**Improving treatment options and the development of targeted therapies**

In part two of the introduction (chapter 1), the addition of targeted therapies to the current treatment options is described. Targeted therapies, aiming to selectively target the tumour with sparing of normal healthy tissues, have the potential to improve the prognosis of the individual patient. However, the development of novel anticancer drugs, including targeted therapies, is an inefficient and very expensive process, with only 10% of drugs entering clinical trials ultimately reaching the market. It is important to obtain clinical information at an early stage of drug development in order to improve the effectiveness of drug development. In chapter 7, the first clinical microdosing (“phase 0”) study ever using immuno-PET as a tool to obtain initial information about the biodistribution, pharmacokinetics, and tumour targeting of the anti-angiogenesis mini antibody F16SIP, is presented. This mini antibody, which is directed against the extradomain A1 of tenascin-C, is considered to be a very promising antibody for antibody-based therapeutic anti-cancer strategies. In this trial, a microdose of F16SIP was labelled with the positron emitter iodine-124 ($^{124}$I, $\tau_{1/2}=100.3$ h) to allow for PET imaging. Information about above mentioned aspects could safely be obtained in a very limited number of patients ($n=4$), and demonstrated limited interpatient variability with tumour targeting in all 4 patients. The tumour-to-blood ratio was about 8 at 5-7 days after injection. Therefore, the conclusion of this study was that further clinical evaluation of F16SIP based therapeutic strategies should be encouraged.

Immono-PET may be considered as one of the most valuable tools for early selection of high potential monoclonal antibodies at an early stage of drug development. Using immuno-PET, drug selection may become more efficient and costs may be reduced. Another aspect is the safety of the patients enrolled in clinical phase I studies. Information obtained from microdosing biodistribution studies might result in less (serious) adverse events by anticipating potential expected adverse effects. In this way, patient discomfort in such trials may be reduced. Future microdosing immuno-PET studies will learn more about whether such studies will indeed speed up drug development.
Summary, discussion and future perspectives