CHAPTER 10

Summary and future perspectives

Summary

Cancer remains a leading cause of death worldwide. Over the last decades many molecules and key pathways in cancer were identified, which shifted anticancer drug development from DNA-damaging chemotherapy towards more personalized molecularly targeted drugs. Many of those drugs are in development, of which (modified) monoclonal antibodies are an important group, as they are highly specific for one antigen and usually exhibit desirable safety profiles.

Reliable biomarkers are needed to enrich the patient population and to determine drug effects in early stages of development. Traditional biomarker analysis for molecular characterization of tumors is performed with immunohistochemistry (IHC) or quantitative polymerase chain reaction on tumor biopsies. However, biopsies are not always feasible and are limited due to accessibility, invasiveness and procedural risks. Moreover, biopsies provide only static information while tumor heterogeneity, including intra-patient and intra-tumor heterogeneity and even changes over time as well as between primary and metastatic tumor lesions, is disregarded.

Molecular imaging is an interesting potential complementary molecular analysis technique. Antibodies can be used as tracers for positron emission tomography (PET) imaging, then called immunoPET. ImmunoPET can noninvasively visualize the presence of specific targets, for which the antibody is developed. Hereby, it can provide whole-body information about tumor uptake and biodistribution of (modified) therapeutic antibodies. Furthermore, it can show whether the drug reaches and accumulates in different tumor lesions in a homogeneous or heterogeneous manner. ImmunoPET can facilitate the development of these new (modified) antibodies by selecting the right patients, determining drug effects in early stages of development and supporting the determination of a rational dose and the optimal schedule of administration. Additionally, it can aid in tumor staging of cancer patients, and can be used as biomarker for response.

Nuclear antibody imaging will always have limitations imposed by the inevitable radiation burden. Although not an alternative for whole-body nuclear imaging, antibodies can also be visualized with optical imaging, when they are labeled with a fluorescent probe. Optical imaging could play a role in drug development by microscopic evaluation of target engagement and modulation and is of interest for surgical guidance.

This thesis aimed to evaluate the role of molecular imaging with radioactive or fluorescent labeled monoclonal antibodies as a biomarker in oncology. We investigated the use of antibody imaging in the treatment of different cancer types, to predict treatment efficacy and to guide decision making in early clinical drug development and during primary surgery.

Chapter 1 provides a general introduction of the topic and outlines the thesis. In **chapter 2**, we presented an overview of the translational process of tracer development. The first step is the determination of an interesting target with an available targeting antibody

and a compatible radioisotope. After labeling the radioisotope to the antibody via a multistep procedure, the radiolabeled antibody has to be validated *in vitro* and in human tumor bearing mice to show the efficiency of tumor targeting. Next, to implement the preclinical developed tracer into clinical trials, a radiopharmaceutical/investigational medicinal product (IMP) has to be produced under current good manufacturing practice (cGMP) guidelines. Thereafter, quality control data, data from non-clinical studies and, if available, prior clinical data, are collected in the investigational medicinal product dossier (IMPD). Finally, the IMPD is required for approval of clinical trials by the authorities in the EU for start of a clinical trial.

Multiple clinical trials have been performed using ^{111}In and ^{89}Zr labeled antibodies for single photon emission computed tomography (SPECT) or PET respectively. Robust trials need to be performed to compare presence of the target in tumor lesions with immunohistochemical staining of a biopsy with the molecular whole-body tumor profile using ^{89}Zr-immunoPET.

In **chapter 3** we reviewed the available literature and status of clinical trials regarding the potential of immunoPET to improve the process of early anticancer drug development. More than 50 antibodies, including several antibody-drug conjugates are in advanced clinical development, forming an important part of the many molecularly-targeted anticancer therapeutics currently in development. Drug development is a relatively slow and expensive process, limiting the number of drugs that are brought into late-stage trials. Development decisions could benefit from quantitative biomarkers to visualize the tissue distribution of (potentially modified) therapeutic antibodies, to confirm effective whole-body target expression, engagement and modulation, and to evaluate heterogeneity across lesions and patients. Such biomarkers may be realized with immunoPET. This approach can potentially increase the power and value of early trials by improving patient selection, optimizing dose and schedule, and rationalizing observed drug responses.

The membrane bound glycoprotein mesothelin (MSLN) is a highly specific tumor marker that is currently exploited as target for drugs. There are only limited data available on MSLN expression by human tumors. In **chapter 4** we aimed to determine overexpression of *MSLN* across different tumor types with functional genomic mRNA profiling (FGM profiling). FGM profiling is a technique that allows prediction of biologically relevant overexpression of proteins from a robust data set of mRNA arrays. This technique was used in a database comprising 19,746 tumors to identify for 41 tumor types the percentage of samples with an overexpression of *MSLN*. Additionally, a literature search was performed to compare the genetic data with studies reporting immunohistochemical (IHC) MSLN tumor expression.

FGM profiling showed *MSLN* overexpression in gastrointestinal (12-36%) and gynecological tumors (20-66%), non-small cell lung cancer (21%) and synovial sarcomas (30%). The overexpression found in thyroid cancers (5%) and renal cell cancers (10%) was not yet reported with IHC analyses. We observed that *MSLN* amplification rate within esophageal cancer depends on the histotype (31% for adenocarcinomas versus 3% for

squamous-cell carcinomas). Subset analysis in breast cancer showed *MSLN* amplification rates of 28% in triple-negative breast cancer (TNBC) and 33% in basal-like breast cancer. Further subtype analysis of TNBCs showed the highest amplification rate (42%) in the basal-like 1 subtype and the lowest amplification rate (9%) in the luminal androgen receptor subtype. In conclusion, FGM profiling reflected known IHC data and moreover showed overexpression in two new tumor types. In this era of individualized treatment strategies, this is of interest for drug development in these tumor types.

MSLN is an interesting target for antibody-drug conjugates (ADCs) especially for the treatment of pancreatic cancer. Noninvasive visualization of an anti-MSLN antibody is of interest to assess biodistribution, quantify antibody uptake in tumor lesions and relate this to antitumor effects. In **chapter 5** we developed a ^{89}Zr-labeled anti-mesothelin antibody (AMA) to study its biodistribution in human pancreatic tumor-bearing mice. First, biodistribution and dose-finding of ^{89}Zr-AMA were studied in mice with subcutaneously xenografted HPAC. Second, microPET imaging was performed at 24, 72, and 144 hours postinjection in mice bearing HPAC or Capan-2 tumors. We additionally performed fluorescence microscopy using IRDye800CW-labeled AMA. Tumor uptake was specific as ^{89}Zr-AMA uptake was higher than control ^{111}In-IgG uptake. With higher doses of AMA, tumor-to-blood and tumor-to-muscle ratios decreased, indicating dose dependent and saturable tracer distribution. MicroPET showed increasing tumor uptake over time in both HPAC and Capan-2 tumors, whereas activity in blood pool and other tissues decreased. Fluorescence microscopy revealed IRDye800CW presence in the cytoplasm of tumor cells, indicating internalization of the tracer. These findings highlight the ability of ^{89}Zr-AMA PET to provide noninvasive, real-time information about AMA distribution and tumor targeting and argue for an expansion of ongoing efforts to use PET imaging in the development of ADCs.

In **chapter 6** we performed the subsequent clinical immunoPET study using the same anti-MSLN antibody as in the preclinical imaging study (^{89}Zr-MMOT0530A) that is part of an ADC (DMOT4039A) in phase I development. We aimed to determine antibody tumor uptake, whole-body distribution and the relation between immunoPET uptake, MSLN expression and response to DMOT4039A treatment. Patients eligible for the phase I study received before DMOT4039A treatment 37 MBq ^{89}Zr-MMOT0530A, followed by PET/CT scans at 2, 4, and 7 days postinjection. Tracer uptake was expressed as standardized uptake value (SUV). IHC MSLN expression was determined in archival tumor tissue.

Eleven patients were included, 7 with unresectable pancreatic cancer and 4 with platinum-resistant ovarian cancer. IHC MSLN expression varied from absent to strong. Suitable tracer antibody dose was 10 mg MMOT0530A and the best imaging time was 4 and 7 days postinjection. Tumor tracer uptake occurred in 37 lesions with mean SUV_{max} of 13.1 (± SD 7.5) on PET 4 days postinjection, with 11.5 (± 7.5) in (n=17) pancreatic and 14.5 (± 8.7) in (n=20) ovarian cancer lesions. Within patients, a mean 2.4-fold (± 1.10) difference in uptake between tumor lesions existed. Uptake in blood, liver, kidneys, spleen, and intestine reflected normal antibody distribution. Tracer tumor uptake was correlated to

IHC. Best response to DMOT4039A was partial response in one patient. This study showed that ^{89}Zr-MMOT0530A PET is able to visualize pancreatic and ovarian cancer lesions as well as antibody biodistribution. The technique has the potential to guide individualized antibody-based treatment.

Thereafter, in **chapter 7** we present the data from the phase I study with DMOT4039A, a humanized anti-mesothelin monoclonal antibody conjugated to the anti-mitotic agent monomethyl auristatin E (MMAE), which was given to patients with pancreatic and ovarian cancer every 3 weeks (0.2-2.8 mg/kg; q3w) or weekly (0.8-1.2 mg/kg). A 3+3 design was used for dose escalation followed by expansion at the recommended phase II dose (RP2D) to evaluate safety and pharmacokinetics. Antitumor response was evaluated per RECIST 1.1 and serum CA19.9 or CA125 declines. Tumor mesothelin expression was determined by IHC. Seventy-one patients (40 pancreatic cancer; 31 ovarian cancer) were treated with DMOT4039A. For the q3w schedule (n=54), the MTD and RP2D was 2.4 mg/kg, with dose-limiting toxicities of Grade 3 hyperglycemia and Grade 3 hypophosphatemia at 2.8 mg/kg. For the weekly schedule (n=17) the maximum assessed dose was 1.2 mg/kg. Due to toxicities limiting re-treatment in later cycles, further dose escalations was deferred and the RP2D level for the weekly regimen lowered to 1 mg/kg. Across both schedules, the most common toxicities were gastrointestinal and constitutional. Drug exposure as measured by antibody-conjugated MMAE and total antibody was generally dose-proportional over all dose levels on both schedules. A total of 6 patients had confirmed partial responses (4 ovarian; 2 pancreatic) with DMOT4039A. DMOT4039A demonstrated evidence of antitumor activity with an acceptable safety profile; therefore, therapeutic targeting of mesothelin represents a feasible approach in the treatment of pancreatic and ovarian cancer.

A relatively novel ADC for the treatment of patients with human epidermal growth factor receptor (HER)2-positive metastatic breast cancer (mBC) is trastuzumab-emtansine (T-DM1) for which only HER2-status determined by IHC and fluorescence in situ hybridization (FISH) has been validated to predict its efficacy. In **chapter 8** we explored the use of ^{89}Zr-trastuzumab-PET (HER2-PET) and FDG-PET to assess intra- and inter-patient heterogeneity in HER2-mapping of metastatic disease and to identify patients unlikely to benefit from T-DM1. HER2-positive mBC patients scheduled for T-DM1 underwent a pre-treatment HER2-PET/CT, and FDG-PET/CT was performed at baseline and before the second cycle of T-DM1. Negative and positive predictive values (NPV, PPV) of HER2-PET/CT, early FDG-response and their combination were assessed to predict morphological response (RECIST 1.1) after 3 T-DM1 cycles and time to treatment failure (TTF). In the 56 patients analyzed, 29% had negative HER2-PET/CT while intra-patient heterogeneity was found in 46% of patients. Compared to RECIST 1.1, respective NPV/PPV were 88%/72% for HER2-PET/CT and 83%/96% for early FDG-PET/CT. Combining both techniques accurately predicted morphological response (PPV and NPV:100%) and discriminated patients with a median TTF of only 2.8 months (n=12, 95% CI: 1.4-7.6) from those with a TTF of 15 months (n = 25, 95% CI: 9.7-not calculable). Therefore, pre-treatment imaging of HER2-targeting, combined with early metabolic response assessment holds great promise

for improving the understanding of tumor heterogeneity in mBC and for selecting patients who will or will not benefit from T-DM1.

Besides nuclear imaging also optical (fluorescence) imaging is of growing interest in oncology, due to its favorable characteristics such as absence of ionizing radiation, intrinsically inexpensive technology, and its possibilities for real-time, intra- and post-operative imaging. In **chapter 9** we report a feasibility study in primary invasive breast cancer patients using the near-infrared fluorescent (NIRF) tracer bevacizumab-IRDye800CW targeting vascular endothelial growth factor (VEGF)-A in which we aimed to provide proof of principle of safety, tumor specific uptake and positive tumor margin assessment. Twenty patients eligible for primary surgery received 4.5 mg bevacizumab-IRDye800CW as intravenous bolus injection. Safety aspects were assessed as well as tracer uptake and tumor delineation *in vivo* during surgery and *ex vivo* in surgical specimens using an intraoperative real-time optical imaging camera. *Ex vivo* multiplexed histopathology analyses were performed for evaluation of biodistribution of tracer uptake and co-registration of tumor tissue and healthy tissue.

None of the patients experienced clinically relevant adverse events. Tracer levels in tumor tissue were higher compared to those in the tumor margin ($P < 0.05$) and healthy tissue ($P < 0.0001$). Also, VEGF-A tumor levels correlated with tracer levels ($r = 0.63$, $P < 0.0002$). All but one tumor showed specific tracer uptake. Two out of twenty surgically excised lumps contained positive margins detected by fluorescent macroscopy of the excision specimen, which were confirmed at the cellular level. *In situ* intraoperative tumor margin detection was not possible, because the administered (micro-) dose was low. Our study shows that systemic administration of the bevacizumab-IRDye800CW tracer is safe for human administration in breast cancer guidance and confirms *ex vivo* tumor(margin) uptake. The findings serve as a step-up towards a phase II dose-finding study aimed at *in vivo* margin assessment and point to a novel tool for drug assessment delivering in-depth tumor tissue distribution.

Discussion and future perspectives

ImmunoPET with the 'naked' antibody of an antibody-drug conjugate in drug development and for patient selection

We performed a clinical immunoPET study using the 'naked' antibody (MMOT0530A) of an ADC (DMOT4039A) for ^{89}Zr-PET imaging before the phase I study with DMOT4039A in pancreatic and ovarian cancer patients. We showed that this technique can visualize and quantify pancreatic as well as ovarian tumor lesions, next to organ distribution.

The ability to safely and accurately predict the presence or absence of the target and binding of an ADC to its target is helpful in the early development of ADCs. The delivery to the target is especially dependent on tissue expression patterns, which can be heterogeneous between tumor lesions within an individual patient. Consequently, some

lesions will be effectively targeted by the drug, while others remain untreated and will contribute to poor clinical outcomes. Comprehensive lesion assessment might provide a useful support, but this approach is impractical with invasive techniques, especially when serial assessment is required. As a noninvasive procedure, immunoPET does not have this disadvantage. The processes responsible for obtaining tumor signal in immunoPET imaging are the same as those for drug delivery by an antibody-drug conjugate: a combination of tissue exposure, tissue penetration, expression of target receptor, and antibody internalization. Therefore immunoPET tracers consisting of the 'naked' antibody of an antibody-drug conjugate can be used for imaging.

In a phase II study with the novel, recently approved, HER2-targeting ADC adotrastuzumab emtansine (T-DM1), we explored the use of ^{89}Zr-trastuzumab-PET (HER2-PET) and early FDG-PET to assess intra- and inter-patient heterogeneity in HER2-mapping of metastatic disease and to identify patients unlikely to benefit from T-DM1. The interim, patient-based analysis showed (compared to RECIST 1.1) a respective negative predicted value (NPV) and positive predictive value (PPV) for HER2-PET/CT of 88%/72% and of 83%/96% for early FDG-PET/CT. Combination of both techniques accurately predicted response with PPV and NPV of 100% and discriminated patients with a median time to treatment failure (TTF) of only 2.8 months from those with a TTF of 15 months. The interim analysis of this study emphasizes that pre-treatment imaging of the target of an ADC (potentially combined with early metabolic response assessment using FDG-PET) holds great promise for improving the understanding of tumor heterogeneity in cancer patients and for selecting patients who may benefit from the ADC in question. Moreover, when heterogeneity exists within a patient, lesions with low antibody-PET uptake can be identified that may benefit more by local treatment. Lesion-based analysis of this ongoing study is therefore eagerly awaited.

Optical imaging in patients is feasible using near-infrared fluorescent labeled antibodies
We showed that systemic microdose administration of fluorescent bevacizumab-IRDye800CW is a safe strategy for fluorescent imaging in patients. Moreover, tumor specific uptake of the tracer was shown by *ex vivo* imaging and microscopic analyses. *In vivo* imaging during surgery may benefit from higher dosage of the tracer, to gain higher tumor-to-background ratios in order to assess tumor margins intraoperatively. Currently, a dose escalation study is being performed assessing the optimal tracer dose for different settings such as breast, esophageal and colorectal cancer surgery and endoscopy (polyps, esophageal and colorectal cancer). Additionally, the intraoperative camera system is being improved with the current knowledge obtained from this study.

Moreover, options are immense in varying with different targets for different tumor types. This may ultimately lead to a mixture of tracers targeting the most frequently overexpressed molecules in a certain tumor type to assess not only target expression for visualization of tumor margins, but also for development of drugs against those targets and assessing their effects.

Functional genomic mRNA profiling to determine target overexpression for molecular imaging

We showed the potential of functional genomic mRNA profiling (FGM profiling) to determine *MSLN* expression across 41 different tumor types. When compared to historical IHC data, the patterns of overexpression were largely comparable, however the percentages of tumor samples with MSLN overexpression differed due to varying cut off points. Currently IHC is often used as a semi-quantitative method for protein expression, but due to the use of different staining antibodies and scoring systems, expression is difficult to compare across different tumor types between studies. This precludes a general cut off for presence of overexpression of certain targets for IHC. With FGM profiling, the cut off can be set at any desired level but is always compared to baseline expression on normal tissue and therefore provides a superior cut off method. For this aim warehouses are filled with large amounts of tumor types that can be relatively quickly analyzed for the existence of a potentially relevant target. Information regarding tumor types and frequency of target presence is of interest for picking relevant targets for therapy.

Antibody imaging as biomarker

In conclusion, this thesis describes various examples of antibodies targeting important tumor targets (HER2, mesothelin, VEGF-A) in clinical development that could benefit from imaging of the antibody using PET or optical imaging. Hereby, information regarding tumor characteristics, biodistibution of the drug, relation with tumor response and IHC scoring can be collected, which aids in understanding the tumor behaviour. Eventually it may even support the prediction of clinical responses and cancer surgery.