Multimodal image-guided interventions using oncological biomarkers

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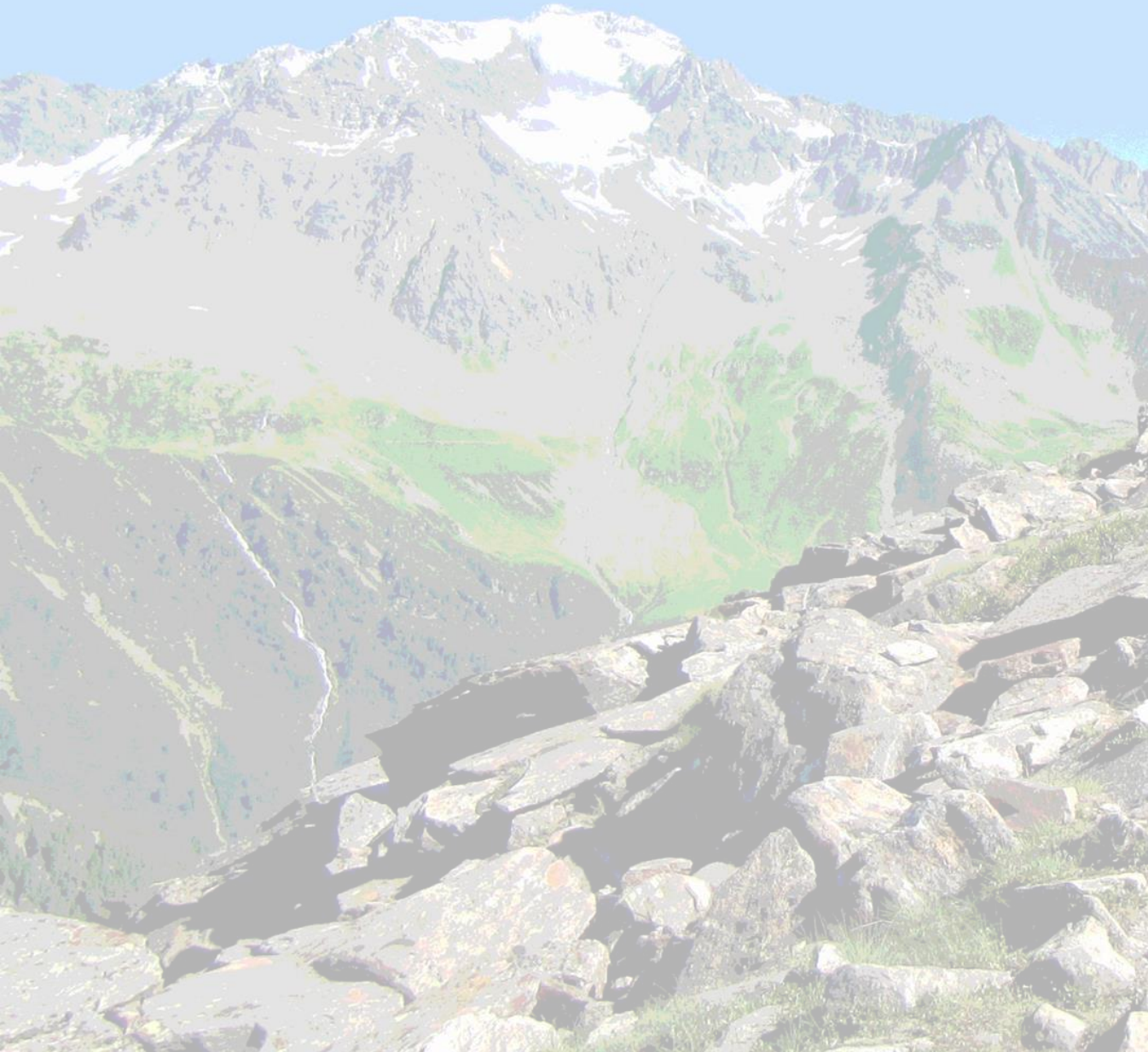

Chapter 8

General discussion, summary, and future perspectives

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This thesis consists of two parts addressing novel imaging technologies to improve the treatment of cancer patients. In part I, the additional value of real time image guidance during surgery is discussed and the research described in this part of the thesis showed that imaging performed during surgery can be of great value. Nevertheless, the success rate is highly dependent on the choice of imaging modality and biomarker to be targeted. In part II, a necrosis avid probe was successfully evaluated as novel method for early neoadjuvant treatment response monitoring.

Part I: Image-guided Surgery

For patients with a solid tumor, extensive diagnostic procedures are performed before the start of treatment as baseline and to determine the cancer stage[1,2]. However, during surgery, the tissue is deformed and margin assessment is only accessible by visual inspection and palpation[3]. In surgical oncology, clear demarcation of the tumor boundaries is off course essential. Nevertheless, the final conformation whether visual tumor resection indeed resulted in a complete tumor resection, can only be determined by final pathology assessment of which the results become available approximately one week after surgery[3]. The main advantage of using (molecular) image-guidance during surgery is to enhance the visualization during the surgical procedure and to provide direct feedback.
In **Chapter 2**, conventional imaging modalities and a variety of state-of-the-art image- and molecular guided surgery modalities are described and compared. The modalities are divided in four groups: conventional, fluorescence, radioactive, and endogenous reflectance. The majority of these techniques encountered a challenge, such as lack of functional information, limited penetration depth or the need for a specifically targeted contrast agent. Unfortunately, there is not a single modality which is able to cover all our clinical needs. Therefore, it is necessary to combine imaging modalities to obtain the best surgical outcome.

Creating sufficient tumor-to-background contrast is one of the main challenges for advanced imaging. In general, imaging contrast is based on endogenous tissue contrast which is not sufficient to detect microscopic involvement of the tumor. The amount of contrast generated in an image can be increased with the use of an exogenous contrast agent. Non-specific exogenous contrast agents, such as gadolinium for MRI or iodine for CT, are

widely available, however, only specifically increase the visibility of the vasculature. As these contrast agents are not tumor-specific, they are only helpful to a certain extent [4-7]. Therefore, there is a need for targeted exogenous contrast agents, which are more specific for imaging of cancer. Nevertheless, the difficulty will be to characterize an appropriate target which is suitable for a defined group of cancer patients [8].

In general, every specifically activated, expressed or upregulated marker on the surface of a tumor could serve as a target for image guided surgery (IGS) [4,9]. Many markers are in different phases of clinical development, of which several show positive results in preclinical investigations. However, the next step, the actual translation from bench to bedside is demanding. Therefore, most probes are not able to pass the early stages of clinical translation, often due to lack of efficacy by detecting a high rate of unspecific binding [7,10-12].

In **Chapter 3** the molecular expression of a target was determined in a human tissue microarray (TMA) to determine whether this would be an interesting target for IGS or not. Using this approach, EphB4, a tyrosine kinase receptor binding the transmembrane ephrin-B2, was identified which is overexpressed in the majority of colorectal cancer patients. Eph receptors and their ephrin ligands play an essential role in cell communication and EphB4 upregulation is associated with cancer progression [13]. To (semi-)quantify the expression of EphA2 and EphB4 in tumor tissue, compared to the expression in adjacent healthy tissue, a normal-to-tumor scorings diagram was developed. This shows that both EphA2 and EphB4 were overexpressed in the majority of colorectal cancer patients. Only EphB4, demonstrated a clear difference between tumor and normal adjacent tissue. Although EphA2 is currently on the prioritization list of the national cancer institute (NCI) to be used as cancer vaccine target, it turned out to be an unsuitable target for the determination of tumor boundaries during surgery [9].
To overcome the drawback of low specificity rates, it is essential that there is a clear difference between expression of the marker in the tumor, as visualized in the scorings diagram, compared to adjacent healthy tissue which will lead to a difference in uptake of the imaging agent. In general, during surgical procedures, a tumor-to-background ratio (TBR) of around 2 is determined as acceptable to provide sufficient diagnostic accuracy [14].

The main aim of **Chapter 4** was to determine whether a combination of pre-operative multispectral optoacoustic tomography (MSOT) and fluorescence guided surgery (FGS) would be able to overcome the drawbacks of limited depth penetration of FGS. This combination provided detailed visualization of an integrin targeting near infrared fluorescent (NIRF) contrast agent, which resulted in a complete and specific overview, both before and during surgery, of the distribution and localization of a pancreatic ductal adenocarcinoma (PDAC) in an orthotopic mouse model. Overall, this chapter clearly showed the additional value of 3D imaging over 2D imaging, independent of the technique used, and showed that MSOT might be a suitable addition or alternative for FGS to improve visualization at a penetration depth over 1 cm.

Part I of this thesis addressed the advantages of IGS. There are, however, also some drawbacks. The disadvantages are related to the use of certain imaging modalities during surgery. The use of a nuclear imaging modality, for instance PET and SPECT, requires the use of radioactivity or, when using tracers with a short half-life, the availability of an on-site cyclotron facility [15]. Also, the magnetic field associated with the use of an MRI requires additional safety and logistic planning requirements, which makes these techniques less attractive and expensive to be used during surgery [16].

Part II: Necrosis Imaging

Cell death is a universal process in the human body and tumor cell death is in general an effect of anti-cancer treatment, which is necessary to cure cancer patients[17]. Cell death is related to the cancer hallmark “resisting cell death” which makes visualizing the amount of cell death via a molecular pathway an interesting concept instead of focusing on morphology [18]. In general, morphological imaging is suitable when the tumor is clearly responding (e.q. shows a reduction in tumor size). However, when this is not the case, anatomical imaging modalities have limited value in differentiating tumor progression from pseudo-progression, which some neoadjuvant therapies can initiate [19,20]. Patients are, in general, only selected to undergo (organ preserving) surgery, after neoadjuvant therapy, when the tumor is responding. When the surgeon is not able to distinguish non-responders from responders it is impossible to select the appropriate patients for surgery [21].

In the second part of this thesis the hypothesis is being explored that an increase in tumor cell death is a sign that a patient does respond to neoadjuvant therapy. Nevertheless, it is also possible that due to the therapy, the biological systems around a tumor are activated to release tumor promoting factors instead of suppressive by which the tumor can grow even faster [22]. In fast growing tumors, angiogenesis cannot keep up with the size of the tumor which will also create an increase in cell death in the core of the tumor. Next to this, it is highly likely that the cell death which occurs after cancer treatment is a combination of several types of cell death and is not solely based on a form of apoptosis or necrosis [23,24].

Imaging cell death

Imaging of cell death nowadays can roughly be divided into apoptotic imaging and necrosis imaging. Apoptosis is a controlled type of cell death, visualizing apoptosis is based on targeting a marker in the apoptotic pathway. Caspases are potential targets nonetheless often difficult to reach as they reside inside the cell [25]. The most well-known apoptosis targeting agent is Annexin V, which selectively binds, with a high affinity, to Phosphatidylserine (PS). Annexin V, labeled with Techneticum-99m, was used in clinical trials, though facing some drawbacks. Suboptimal biodistribution patterns have been found with a high background uptake in the abdomen. Another disadvantage is that PS as target is only available for a limited amount of time in the process of apoptosis. An alternative target is phosphatidylethanolamine (PE) targeted via duramycin. The major advantage of PE over PS is the higher availability of PE on the cell membrane. Duramycin as probe is stable with a high binding affinity and specificity, radiolabeled with Techneticum-99m makes it a promising probe, currently available for preclinical studies [25,26]. Although apoptosis imaging will probably be able to reflect treatment response as the amount of apoptosis is relative high, it is not tumor specific as it targets a process which also is required to occur in a healthy multicellular environment to maintain homeostasis [17]. Therefore, the accuracy to identify tumors with a marginal treatment response might be limited.

Necrosis imaging is also not tumor specific. The chance of coexistence of a simultaneously ongoing pathological process of cell death in the neighborhood of the tumor is quite unlikely. This makes necrosis an interesting target for tumor treatment response evaluation. Probes targeting necrosis can be

divided in three groups: porphyrins, antibodies and dianthrones. The first group consists of gadolinium-diethylenetriaminepentaacetic acid (Gd-DTPA) derivatives which accumulate in nonviable tissue. Unfortunately, porphyrins are hampered for clinical use mostly due to their phototoxicity, limited effectiveness and difficult synthesizing procedure [27-29]. Antibodies, the second group, are in general more specific. One of the antibodies, Cotara, is directed against the DNA histone complex which is present in dead and dying cells in the center of solid tumors [30,31]. Another antibody, Myoscint, targets the intracellular heavy chain of myoscin. In general, the clinical translation of antibodies is challenging due to several reasons including their size which results in a limited tissue penetration due to disturbed architecture of the vasculature [32].

The last groups are dianthrones, Hypericin, is a natural occurring photosensitizer, proved to be a potent agent for photodynamic therapy in cancer treatment and also shows necrosis avidity. Hypericin and its derivatives Sennidin A and Sennoside b are labeled with Iodine for both diagnostics and therapeutic approaches. The main drawback of these agents is that they are phototoxic and difficult to dissolve [25,33-35].

The carboxylated cyanine dyes tested in this thesis do not suffer from the drawbacks of the above-mentioned agents, as they are non-toxic small molecules which are easy to dissolve.

Therapeutic cell death

The most prominent process of cell death, initiated by chemotherapy and radiotherapy, was investigated to prove the value of our necrosis avid contrast agent. Chemotherapy and radiotherapy were chosen as they are the two most frequently used approaches of neoadjuvant therapy [36,37].

Chemotherapy induces alterations in Adenosine Triphosphate (ATP) levels, which is distinctive for dying cells: ATP decreases intracellularly and increases extracellularly. The level of decrease intracellularly will determine the switch between apoptotic or necrotic cell death. When cell death progresses and the membrane potential is completely disrupted, the intracellular ATP level is further decreased, finally leading to secondary necrosis. Based on this knowledge, apoptosis is the initial form of cell death after treatment with a chemotherapeutic agent, followed by (secondary) necrosis [17,38]. However, some chemotherapeutic agents, such as cyclophosphamide, induces type I immunogenic cell death (ICD). Cyclophosphamide is used in the experiments

in **Chapter 5 & 6,** in an immunodeficient mouse model. ICD is a form of cell death which is closely related to the secretion and release of damage-associated molecular patterns (DAMPs) [39,40]. This means that molecules inside the cell, which under normal circumstances are not associated with immunological functions, will be released, secreted or exposed on the cell surface of damaged or dying cells. In this way, they trigger an immune response in the absence of infection and they stimulate immunogenicity through endoplasmatic reticulum stress related effects [40]. Extracellular ATP is seen as DAMP and as mentioned above the amount of extracellular ATP determines the cell death pathway [39].
Radiotherapy eliminate cancer cells by the use of ionizing radiation [41]. Ionizing radiation creates DNA damage which will have a direct and indirect effect on cells both leading to cell death. Irradiation with a high dose per fraction (>10 Gy) will cause, in general, more direct DNA double strand breaks and will result in lethal damage in a higher number of cells, as compared with irradiation using a lower fraction dose (<10 Gy). Nevertheless, this is compensated with fractionation which will cause a similar linear decrease in the number of surviving cells at the end of the treatment [37,42,43]. In addition, fractions above 10 Gy will also cause severe vascular damage, leading to indirect cell death [42]. Vascular damage causes a reduction in blood perfusion and has a negative influence on the oxygenation status, severe ischemia will lead to necrotic cell death [17,44].

The results described in **Chapter 5** shows that the two identified NIRF carboxylated cyanine dyes, HQ5 and IRDye800CW, possess strong necrosis avidity. The exact molecular targeting mechanism is not clear; however, it involves avidity for probably a mixture of cytoplasmic proteins available after loss of cell membrane integrity.
In preclinical research, the advantages of using optical modalities over radionuclide imaging are clear as they require less safety requirements, are cheap and fast. The downside of limited tissue penetration and lack of quantification is relatively small in early stages of research. Research closer to clinical translation needs quantitative pharmacokinetic and distribution profiles which are demanding to provide with optical imaging only. In addition, the limited penetration depth of only 1 cm makes the technique not feasible for quantitative pharmacokinetic studies in humans.

To enable quantification and a possible clinical translation, in **Chapter 6** one of the members of the family of HQ5 cyanine dyes, HQ4, was conjugated with Indium-111 as radiolabel via the chelate DTPA. This chapter illustrates that also after radiolabeling the necrosis avidity was still intact and could be visualized both with the fluorescence- and the radiolabel. Moreover, due to radiolabeling, the specificity of the probe *in vivo* could be demonstrated. It was shown that the uptake was not solely due to the enhanced permeability and retention (EPR) effect. Latter could be caused by the positive charge of the HQ-compound, conceivably leading to binding to albumin. By comparing the radiolabel alone with radiolabeled HQ4, a significant difference in tumor uptake could be measured.

In **Chapter 7** another imaging modality, optoacoustic tomography, was validated. In this chapter, the performance of the probe was confirmed as a truly multimodal contrast agent for both superficial and deeply situated tumor imaging. Based on the obtained results, the necrosis avid contrast agent, HQ4, has the potential to be clinically translated for multiple purposes. One of those purposes is the evaluation of treatment (chemotherapy and or radiotherapy) response, which was already successfully tested in **Chapter 6,** by monitoring chemotherapy response, at clinically relevant dose levels. **Chapter 7** further elucidates its use in monitoring radiotherapy induced tumor cell death. Nowadays, measuring treatment response is mostly performed with ^{18}F-fluorodeoxyglucose (FDG), the most widely available PET-tracer [45]. In several tumor types, FDG-PET showed a high predictive value to assess tumor response early after start of treatment. The disadvantage of FDG is that it is taken up by cells with an increased glucose metabolism. Macrophages, often involved in the removal of necrotic tumor cells, also accumulate FDG. In addition, it is challenging, during radiotherapy, to discriminate between FDG uptake in tumor cells or radiation-induced inflammation, inducing an underestimation of the treatment response. Nevertheless, it is already proved for both lung and rectal cancer patients treated with chemoradiotherapy that the alteration in glycolysis level or standard uptake value during therapy was predictive for progression-free survival. In addition, scans obtained 3 months post-treatment showed a clear positive correlation between FDG uptake and patient outcome [25,46-50]. Another promising PET-tracer is ^{18}F-fluorothymidine (FLT) which detects cell proliferation; the uptake is positively correlated with cell growth. It has been found that, in preclinical research, FLT is superior to FDG and that it is a sensitive and early predictor of therapy response in various

cancer types [51]. In clinical practice, however, is found that the sensitivity of FLT is lower compared to FDG and that a decrease in uptake during therapy is not associated with a longer overall survival which makes it less suitable to use for treatment evaluation and adaptive therapies [48]. As mentioned above, there are a couple of other promising agents in several stages of clinical development which can also be used for the same purpose. Unfortunately, it is not possible to compare these probes with the results obtained with HQ4 (**Chapter 6 & 7**), since each of the probes visualizes another biological process. Necrosis is the only process which is in general not present in healthy tissue though also not solely linked to cancer.

For longitudinal monitoring of treatment responses most probes could be used as an increased cell metabolism, cell proliferation, apoptosis and necrosis are all taking place in the tumor environment. However, the use of HQ4 is in this form the least favorable, as it is coupled to the SPECT isotope Indium-111, which has a long half-life. SPECT has a lower intrinsic resolution and sensitivity as compared to PET. An isotope with a longer half-life, however, is necessary as it takes about 24h before HQ4 reaches its most optimal TBR. Indium-111 could be replaced by a PET isotope like Zirconium-89 to be able to image it with PET. However, DTPA is not the most optimal chelate to incorporate Zirconium-89, desferrioxamine (DFO) is a better alternative [52]. Nonetheless, HQ4 can be a suitable theranostic agent, since instead of Indium-111, also Ytrium-90 or Lutetium-177 can be incorporated.

Future Perspectives

Combining targets

Over the last years a lot of anticancer drugs have been developed. Unfortunately, only a small percentage reached the clinical market. The same is true for targeted contrast agents. Due to a high level of tumor heterogeneity in human cancer it will be difficult to find one suitable, personalized target which will target the whole tumor. Instead of using one target it is probably more favorable to use a variety of targets combined in one targeted contrast agent. In this way, one could create the potential to target and visualize multiple receptors at the same time. The question which markers to combine is difficult to answer and depends on two main points. The first point is related to the tumor to be imaged. Suggestions would be to combine markers towards targets directed to one of the biological processes mentioned in the "hallmarks of cancer" instead of combining highly specific markers downstream the cascade [18,53]. Additionally, literature already showed that targeting multiple pathways is more effective compared to targeting multiple targets of one pathway, especially when the tumor is trying to bypass a certain pathway, in case of multidrug resistance [10,54]. This also accounts for the development of a broadly applicable targeted contrast agent. Final suggestion would be to combine markers which are known to be upregulated in different phases of the disease development to increase sensitivity. The second point to take into account when combining targets is the chemical construction and pharmacological behavior of such probes: its physicochemical character. This character can be partly predicted by the use of quantitative structure activity relations (QSAR) modelling. Nevertheless, in general, an increase in size and alteration in charge will influence the clearance and biodistribution of a probe [55-57]. In addition, some combinations of targeting moieties or imaging agents can interact with each other by which they could block their function or lose their specificity leading to unwanted high background signals [58].

Combining imaging modalities

As already concluded above there is not a single imaging modality which could be used for all purposes. Combining imaging modalities is an option to circumvent this problem. Unfortunately, the generation of hybrid imaging modalities is only feasible with a limited number of machines. Nevertheless, fusing of the images obtained is always an option. Combining analysis data of different modalities instead of analyzing each modality separately will create a

high-dimensional dataset. New methods to analyze such data sets are under development, like t-distributed stochastic neighbor embedding (t-SNE). T-SNE will give more detailed information and will hopefully reveal new relationships which could be used to stratify patients before they start with their cancer treatment or during neoadjuvant treatment [59]. However, it will be demanding, if ever possible, to use this during IGS, where analyses and feedback need to be performed fast and real-time.

In **Chapter 4** MSOT is introduced to overcome the limited penetration depth of FGS. In this chapter, the preclinical MSOT machine is used, showing promising results, however, it is still limited by relatively long acquisition and reconstruction times. These long acquisition times are necessary due to the limited sensitivity as compared to fluorescence only imaging systems. Nowadays, clinical MSOT systems, used in combination with ICG, are available and the time to capture single cross-sectional images is reduced to less than 1 ms [60]. In addition, the concentration of ICG for SLN detection in melanoma using either MSOT or fluorescence is relatively similar with a dose of 0.5 mg ICG injected around the tumor [60,61]. Hopefully, newer generations of this imaging system will reach higher sensitivity, which is of importance to further increase the resolution, to reduce the dose of the contrast agent and/or to decrease image acquisition time. In addition, as it is impossible to chemically link a targeting agent to ICG, ICG will be replaced in the future for another fluorophore, such as IRDye800CW combined with a targeting agent, which will hopefully improve detection sensitivity even further. The future perspective would then be that MSOT could be used both for image guided surgery and for diagnostic imaging purposes, for instance for treatment monitoring of not only superficially located melanoma but also for deeper-located tumors. Nowadays, for instance, human breast cancer is already visualized via a label-free method, reaching imaging depths of up to 2.5 cm [62]. Nevertheless, reaching an imaging depth of over 5 cm, at least necessary for full clinical usage, will not be reached with this technique so far. However, when MSOT would become available as endoscopic tool, this penetration depth would be sufficient in for example rectal cancer Watch & Wait strategies.

The Watch & Wait strategy offers a lot of opportunities for patients with a complete clinical response after neoadjuvant therapy for rectal cancer and oesophageal cancer patients. When those patients could be identified before

surgery, unnecessary procedures and subsequent postoperative morbidity could be avoided. However, for those patients it is essential to keep them under a strict follow-up regimen to detect possible local recurrences in an early stage [63,64]. During the follow-up period the use of personalised imaging with targeted contrast agents is obligatory. For Watch & Wait PET-CT for total body imaging could be used in combination with an endoscopic modality by either fluorescence or MSOT.

Combining therapy and diagnostics

Theranostics combines diagnostics and therapeutics to eliminate multi-step procedures and increase efficacy by using the diagnostic agent to visualize whether the proposed treatment will arrive at the tumor site [65,66]. By using click chemistry this efficacy can be even further improved as click chemistry combines the beneficial targeting properties of, in general, antibodies to reach high TBRs, with the fast pharmacokinetics of small molecules for therapeutic agents [67,68]. The method relies on a two-step approach in which in the first step an antibody, labeled with a click label, is injected. After a couple of days, when the antibody is accumulated in the tumor and cleared from the blood, the second part is injected consisting out of a radionuclide combined with the opposite site of the click label. As the radiolabel is relative small it will be cleared fast from the blood and does not accumulate in other parts than the tumor [67]. Such an approach is investigated for radioimmunotherapy, however, can also be used for IGS in combination with radionuclide therapy [67,68]. Administer a patient a couple of days before surgery with the first agent, now additionally labelled with a NIR fluorophore to use during surgery. Afterwards the second part is injected to treat possible tumor residues with targeted radionuclide therapy based on the same probe.

To finish, as the Chinese philosopher Lao Tzu quoted; *“the journey of a thousand miles begins with one step”, w*hich means in the context of the present thesis: “*to beat a high variety of cancer types, all knowledge gained is a little step forward in unraveling the behavior of cancer. Each tiny little step will help in the fight against cancer and I hope that this thesis provided such tiny step forward in the right direction!*”

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