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Multimodal image-guided interventions using oncological biomarkers

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Citation

Stammes, M. A. (2018, May 22). *Multimodal image-guided interventions using oncological biomarkers*. Retrieved from <https://hdl.handle.net/1887/62351>

Version: Not Applicable (or Unknown)

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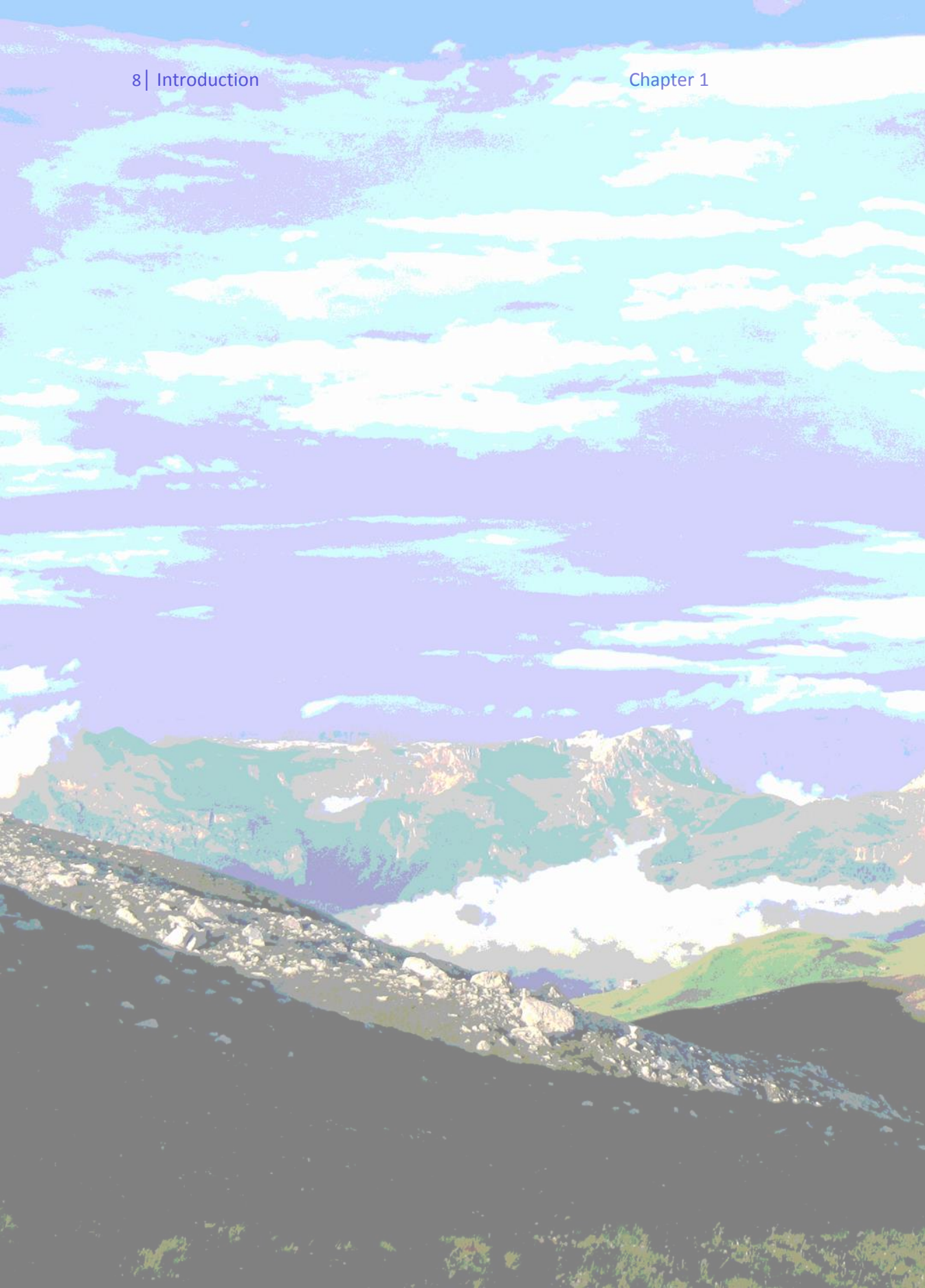


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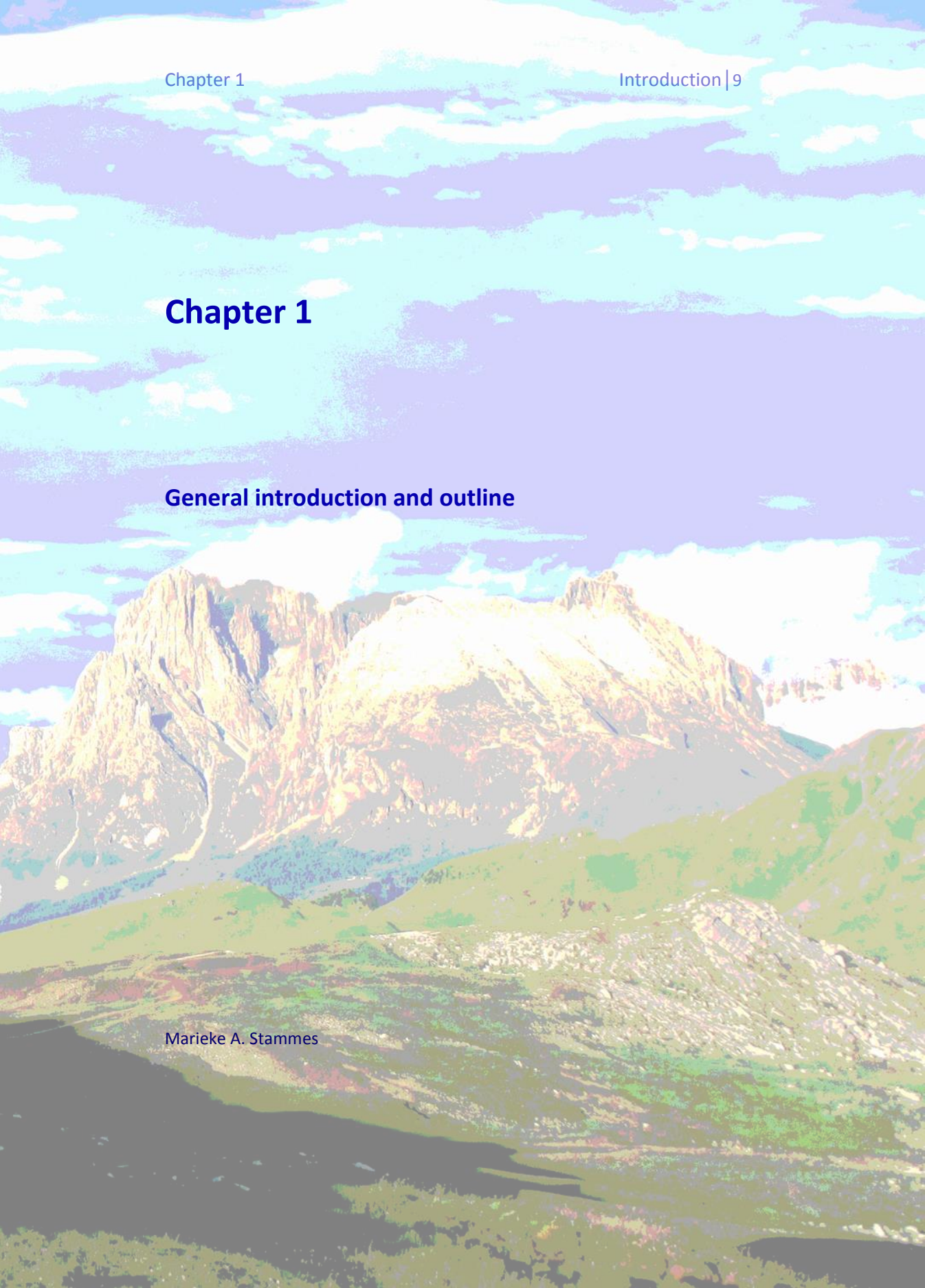
Issue Date: 2018-05-22



Chapter 1

General introduction and outline

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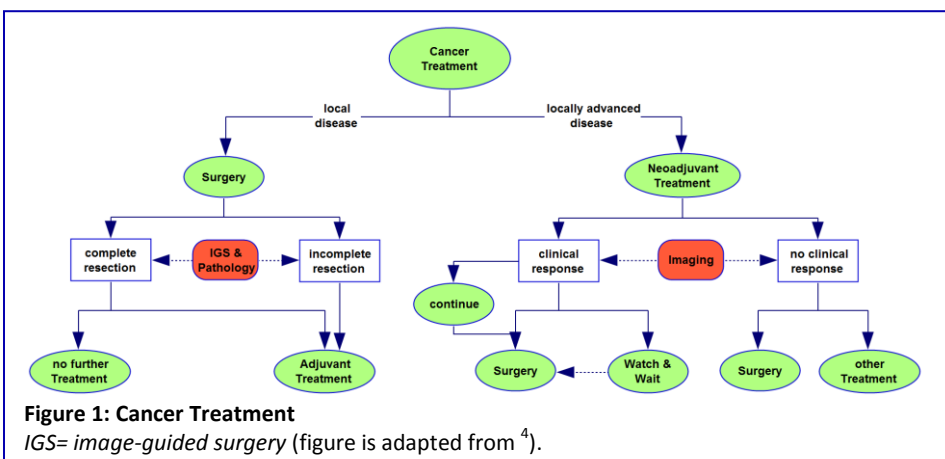


General introduction

Cancer is one of the leading causes of mortality worldwide, with around 14 million new cases in 2012 ¹. It is expected that this number will increase up to 20 million around 2025, which means that the cumulative risk factor of people who will be afflicted with a form of cancer, which is now already 18.5%, will increase further over the upcoming years. The common goal of many research institutes around the world is to decrease the cancer related morbidity and mortality rate ^{1,2}.

Cancer biology

Cancer can be defined as the rapid creation of abnormal cells that have the ability to divide and grow uncontrollably beyond their usual boundaries, after which they can spread and invade in other organs ³. Although this definition is clear, a tumor is more complex than a mass of proliferating cancer cells. It is a heterogeneous and complex disease regulated by genomic alterations. The complexity is due to the different locations and number of alterations or mutations between cancer types and between patients with the same type of cancer. Some mutations will create oncogenes which will work in favor of the tumor and others will lead to a loss of function of tumor suppressor genes. In addition, the transformation from a normal cellular environment into a malignant tumor environment, tumorigenesis, is a multi-step process. This process is reflected by genetic alterations in the regulatory circuits of cells which normally would maintain homeostasis. Although a lot of processes are involved, some molecular, biochemical, and cellular characteristics are shared by most tumors. Those alterations are rationalized by the well-known cancer hallmarks as described by Hanahan and Weinberg ^{6,7}. Unfortunately,



nowadays, these molecular alterations do not play a major role in the treatment decision process.

Cancer treatment

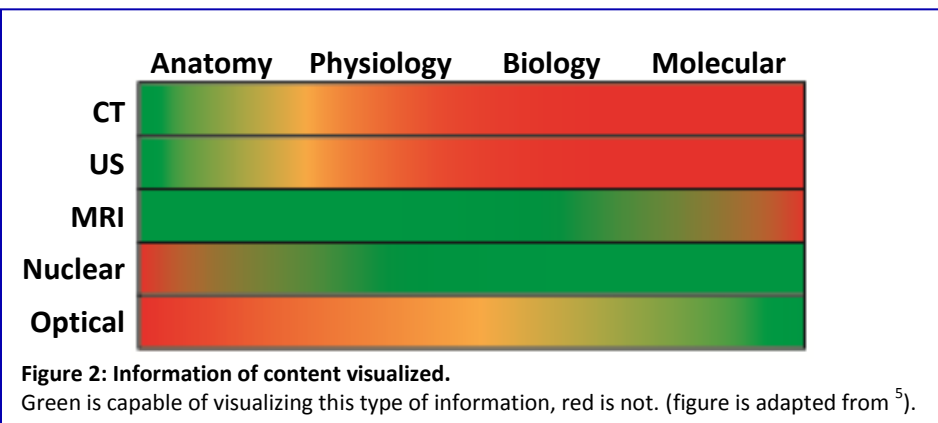
There is no doubt that cancer will remain a significant healthcare problem for the years to come. Fortunately, early diagnosis and improvements in therapy, including neo-adjuvant treatments, have led to serious improvements in survival rates and quality of life. However, due to the fact that there are more survivors, there is also an increase in late toxic side effects which will influence the quality of life in a negative manner ^{8,9}. Surgical interventions, systemic treatments and radiotherapy or a combination thereof, are the main treatment modalities for cancer ¹⁰. Whenever possible, surgery is the first choice to treat localized disease (**Figure 1**) ^{4,11}. In case of locally advanced disease, patients can be offered neo-adjuvant treatment before surgery (as shown in **Figure 1**). The goal of this therapy is to reduce tumor invasion and size of the primary tumor allowing radical or more organ sparing surgery and to improve locoregional disease control ^{12,13}.

Cancer research

Imaging

With the aim of improving the clinical outcome of cancer patients, cancer research is directed towards developing personalized and targeted treatments to increase the specificity in eradicating the cancer cells and sparing healthy tissue ¹⁰. Diagnosis, staging and treatment planning are determined by several techniques, and imaging is indispensable to provide information about the tumor biology and anatomical structures. Frequently used imaging modalities to gain information about the anatomical structures involved are computed tomography (CT), magnetic resonance imaging (MRI), ultrasound (US) and conventional radiography. Although anatomical imaging provides information about tumor morphology, it does not always give functional information about the tumor. Molecular imaging techniques can provide valuable functional information. In general, with molecular imaging it is possible to non-invasively visualize, characterize and measure biological processes of the tumor and the tumor micro-environment at a molecular level ¹⁴. The most well-known and clinical used molecular imaging techniques are single photon emission tomography (SPECT) and positron emission tomography (PET).

For intra-operative imaging, the use of optical imaging is gaining interest and



is nowadays widely used in both clinical and pre-clinical research ¹⁵. The additional value of near-infrared fluorescence in the range of 700-900 nm over lower wavelength fluorescence is a higher tissue penetration of up to a centimeter and low auto fluorescence ¹⁶. The drawbacks are the lack of whole body overview and still the limited penetration depth. Therefore, combining techniques like optical and nuclear imaging can have synergistic effects (**Figure 2**). The same is true for the introduction of PET-CT as hybrid imaging modality. Both single modalities, PET as molecular modality and CT as morphological modality, lack the requirements for an accurate and complete *in vivo* assessment. However, together providing anatomical, physiological, biological and molecular information this problem will be solved ^{5,15}. The specifications of several imaging techniques and the use of hybrid techniques are discussed more in depth in **Chapter 2**.

Targets

For molecular imaging the use of a-specific contrast agent the so-called molecular imaging agent is required as the goal is to assess the expression or function of a dedicated molecular target ^{14,17}. There are two main approaches of developing a targeting agent by conjugation or by structure-mimicking. The first is the most widely used method in which a contrast agent consists out of a targeting moiety associated with the molecule of interest, a contrast-enhancing agent dependent on the type of imaging modality and a linker to connect those two parts. The other approach is to chemically modify a molecule that naturally interacts with the molecule of interest. With this approach a targeting moiety can directly be converted in a contrast agent ¹⁸.

Using a targeted contrast agent is called active targeting, however, passive targeting is also possible via the enhanced permeability and retention effect (EPR) by which molecules leak into the tumor¹⁹. The drawback of passive targeting in combination with surgery is that the targeting is a-specific and, as discriminating the boundary of a tumor is essential during surgery, agents that leak into the core of the tumor are not the best candidates¹⁹⁻²¹.

In general, targets can be divided in a genetic and a mechanistic class. The first represents genes or gene products that carry mutations or that lead to a higher disease risk. In our genome, which consists out of 25.000 genes, around 1800 genes are involved in diseases such as cancer. This means, including post-translational modifications, over 40.000 different proteins are available as possible target²². The second class of targets consists of receptors and enzymes which are, in general, not different from the normal population, they do not have any mutation. However, biological observations detected that their behavior differs from a normal situation. This suggests that they might be involved in a tumor process and by this be an interesting target^{21,23}. When an interesting target is found, an agent is developed towards a binding pocket in the target of interest and, when necessary, afterwards modified to enhance the pharmacokinetics and pharmacodynamics before it could be used as targeted contrast agent²⁴.

The general aim of this thesis is double, first to improve image-guided surgery (IGS) via innovative imaging modalities in combination with targeted contrast agents which will lead to improved intra-operative visualization of resection margins during surgery. Second, for patients who undergo neo-adjuvant therapy, the aim is to ameliorate treatment response monitoring already in an early stage after start of treatment. Therefore, the second part of this thesis will focus on detecting the amount of necrosis, as biomarker, after chemotherapy and/or radiotherapy. The background information of each part is separately discussed below, followed by an overview of the content of the chapters.

Part I: Image-guided Surgery

As mentioned above multiple imaging modalities have proven to be essential for cancer diagnostics, providing much information about the tumor before surgery^{25,26}. However, during surgery, when the anatomy changes due to manipulation, the surgeon relies on palpation and visual inspection to accurately determine whether the resection margins are tumor free²⁷⁻²⁹. In oncologic surgery, clean and clear demarcation of the tumor boundaries is essential for proper removal of the tumor. There is a fine balance between achieving tumor free resection margins and sparing healthy tissue and organ function. Therefore, visualization of the tumor borders with a high sensitivity is essential for an optimal and safe removal of the cancer lesion^{30,31}. The eyes and hands of a surgeon are useful, but cannot determine the boundaries of a tumor at a microscopic level²⁹. Therefore, the risk remains of an irradical resection of the tumor, also known as an R1 resection. For instance, in breast cancer patients, undergoing breast sparing surgery, the percentage of R1 resections is around 20%^{27,28,32,33}. To improve cure rates, it is important to decrease local recurrence rates as well as complication rates. To reach this goal, intra-operative assistance, using additional visualization tools could be helpful. Nowadays, during oncologic surgery intra-operative ultrasound or x-ray fluoroscopy are the only two modalities which provide real-time visualization. Drawbacks of these modalities are the necessity of direct contact with the body, the use of ionizing radiation and the current lack of availability of targeted contrast agents²⁷. The details about the imaging systems are discussed more in depth in **Chapter 2**.

Up to now, in clinical trials, tumor specific optical IGS is already performed with several agents, such as 5-ALA for gliomas³⁴, folate-near infrared (NIR) fluorophore for ovarian cancer^{35,36} and bevacizumab-IRDye800CW (in combination with hyperthermic intra-peritoneal chemotherapy (HIPEC)) for peritoneal carcinomatosis³⁷. Results obtained from these trials are two-sided with promising results and hurdles which need to be overcome for a broader clinical implementation. These studies demonstrated that fluorescence imaging did not interfere with the standard surgical procedure, showed real-time identification of the tumor and was able to reduce overtreatment, due to a higher number of R0 resections. The two main problems encountered are the high amount of false-positive findings of lymph nodes in particular and the limited penetration depth of optical imaging^{34,35,37,38}. For the development of new IGS approaches, there is a need for dedicated targeted contrast agents

with high tissue specificity and a high tumor to background ratio (TBR) for a more detailed visualization of the tumor (**Chapter 3**) in combination with alternative imaging modalities (**Chapter 2 & 4**)^{19,28}.

Part II: Necrosis Imaging

Treatment evaluation

Most systemic anticancer therapies are effective only for a subgroup of patients. Unfortunately, up to now there are only limited possibilities to stratify patients for specific treatments. Therefore, it is difficult to predict upfront which patient will benefit from a certain treatment and which one will not³⁹. Currently, the treatment regimen is based on population based statistics, which have limited value for the individual patient. Evaluation of the treatment is nowadays generally based on measuring tumor shrinkage at a late stage during and after treatment mostly based on the RECIST criteria; Response Evaluation Criteria in Solid Tumors. Those criteria are generally used as end points in clinical trials or for routine clinical decision making in order to decide to stop, continue or switch to another treatment⁴⁰⁻⁴². Nevertheless, monitoring the anti-cancer efficacy of a therapy at an early stage of treatment would have multiple advantages³⁹. Existing methods for the determination of tumor markers in blood lack the accuracy for a broad routine application for treatment response monitoring^{43,44}. Early evaluation of the therapy efficacy would facilitate the growing interest for individualized cancer treatment, allowing the clinician to adjust the therapy based on tumor response and might result in improved survival rates, quality of life and cost-efficacy^{45,46}.

As discussed above, morphologic changes often can only be detected several weeks to months after the start of treatment. Functional and molecular alterations, on the other hand, occur much faster, already in the first weeks after start of treatment. Most of those molecular alterations are in a way related to restoring homeostasis and creating cell death. For this reason cell death is a biological process which can also be used as biomarker of tumor response in the treatment of cancer patients^{39,47}.

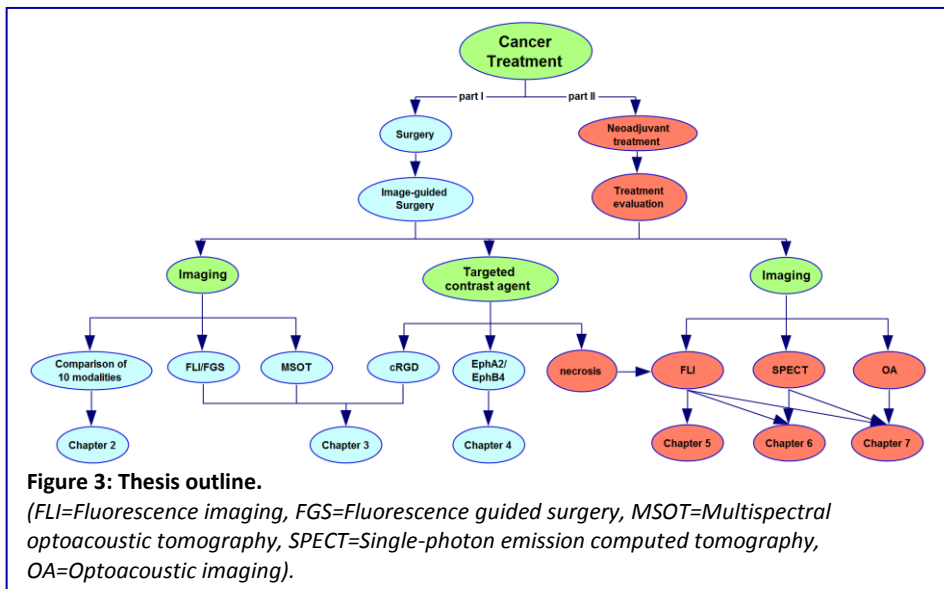
Cell death

Cell death is a process that is reversible until the first irreversible step is made. Different steps could represent this phase but a cell is considered death when

the cell has lost its membrane integrity, undergone complete fragmentation and when the cell corpse is engulfed by an adjacent cell. This diversity leads to a high variety of types of cell death which are classified according to its morphology, enzyme involvement, functional aspects and immunological characteristics⁴⁸. Nevertheless, it is possible to narrow it down to the two fundamental and most well-known types of cell death; apoptosis and necrosis^{39,45,48,49}. In general, apoptosis is required to maintain homeostasis and the main mechanism by which cells die in the human body^{39,49}. Necrosis is the result of metabolic failure and usually occurs in response to acute hypoxic or ischemic injury⁴⁹. Based on the facts above, it implies that the process of cell death in apoptosis and necrosis are clearly distinct from each other. However, when the number of cells in apoptosis is too high for the phagocytes to eradicate them, this will lead to secondary necrosis. Secondary necrosis is also a form of necrosis, although the dying mechanism is still regulated by apoptosis⁵⁰.

In part II of this thesis, the value of intra-tumor necrosis, as unique biomarker, is investigated. Necrosis is used to monitor the anti-cancer efficacy of a treatment at an early stage after start of treatment. Necrotic cell death plays a prominent role in multiple pathological and physiological disorders; especially in cancer as tumor necrosis is associated with tumor aggressiveness and negatively correlated with disease prognosis⁵¹⁻⁵³.

Chapter overview



In **Figure 3** a graphical overview of the thesis is visualized, when comparing this figure to **Figure 1** a close comparison can be found between a cancer treatment and this thesis.

In **Chapter 2**, an overview is provided of the state of the art imaging modalities which can be used intra-operatively. Next to the imaging modalities, mentioned in this thesis, several applications of molecularly IGS will be discussed. The image quality of most of these techniques can be improved by using a dedicated contrast agent. Targets upregulated at the cell surface, abundantly present on tumor cells and absent on surrounding healthy cells are the best candidates to be used for IGS.

In **Chapter 3**, two proteins, the transmembrane receptor tyrosine kinase EphA2 and EphB4, are evaluated as possible targets for IGS in colorectal cancer on a tissue microarray. In this chapter, EphA2 is derived from the National Cancer Institute (NCI) prioritization list and is known to be highly overexpressed on a high variety of tumor types, including colorectal cancer. EphA2 is compared to the family member EphB4 from another class²¹. EphB4, a member of the B class of the same family is upregulated in many solid tumors. However, for both EphA2 and EphB4 their expression in healthy tissue has not been investigated. In this chapter, the TBR of both proteins will be

determined and discussed whether EphA2 and EphB4 are valuable candidates for IGS⁵⁴⁻⁵⁶.

In **Chapter 4** the additional value of a new imaging modality, multispectral optoacoustic tomography (MSOT) is assessed. MSOT uses light as a source and acoustics for signal detection to create images which enables the visualization of optical absorbing agents at a high resolution similar to optical imaging. However, the main advantage of MSOT compared to optical imaging is the accurate spatial localization within deep tissue since the detection is not limited by photon scattering enabling a higher depth penetration^{57,58}. In this chapter, the additional value of MSOT is compared to a couple existing fluorescence imaging modalities. The value and the results obtained with MSOT and the other modalities are validated both *in* and *ex vivo* by using an orthotopic pancreatic ductal adenocarcinoma (PDAC) mouse model targeted by cRGD in combination with the fluorescent dye IRDye800CW⁵⁹⁻⁶¹.

The second part of the thesis will focus on different imaging techniques to visualize tumor necrosis.

In **Chapter 5**, two novel optical necrosis avid contrast agents, IRDye800CW and HQ5 are identified and evaluated. Those contrast agents both belong to the group of carboxylated cyanine dyes which are in general mentioned as non-reactive and control compounds. In addition, IRDye800CW and HQ5 both belong to the group of near-infrared dyes. Their necrosis avidity is validated *in vitro* and *in vivo* in mouse breast cancer tumor models of spontaneous necrosis or necrosis induced by chemotherapy.

In **Chapter 6**, one of the family members of HQ5, HQ4, is equipped with a radioactive moiety to facilitate the clinical translation in the future. Near-infrared dyes have a tissue penetration of up to a centimeter. However, a tissue penetration of one centimeter still limits a broad clinical application. By using radioactivity the penetration depth is unlimited. In addition, with the use of radioactivity, quantitative biodistribution studies were performed to get more insight in the behavior of the agent.

In **Chapter 7**, the knowledge gained in the previous two chapters is further extrapolated by evaluating HQ4 for monitoring tumor cell death induced by radiation therapy in a clinically relevant MCF-7 human breast cancer mouse model. Furthermore, the multimodal imaging properties of the necrosis avid contrast agent were assessed with the addition of a third imaging modality, optoacoustic or photoacoustic imaging. Optoacoustic imaging is similar to MSOT with the only difference that MSOT uses a multispectral illumination

approach in order to differentiate even more specific spectral signatures of exogenous or endogenous contrast agents⁶². This modality is tested because it has the benefit of a higher tissue penetration as compared to optical imaging and it overcomes the drawbacks associated with the use of ionizing radiation.

Finally, in **Chapter 8** a general discussion is provided, including a summary and the future perspectives of multimodal image-guided interventions are addressed.

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