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Exploring novel opportunities for molecular imaging in surgery

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EXPLORING NOVEL OPPORTUNITIES FOR MOLECULAR IMAGING IN SURGERY

ILLUMINATING SURGICAL DECISION MAKING

Pieter J. Steinkamp

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EXPLORING NOVEL OPPORTUNITIES FOR MOLECULAR IMAGING IN SURGERY

ILLUMINATING SURGICAL DECISION MAKING

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CHAPTER 1

General introduction

INTRODUCTION

Precise pre- and perioperative surgical assessment of the individual patient with its surgical treatable disease requires adequate, safe and a stepwise clinical introduction and optimization of easy implementable, sensitive and accurate imaging techniques. These imaging modalities should eventually increase surgical quality and patient outcome improving diagnosis, treatment planning, the surgical procedure and anticipated (dis)advantages for the individual patient.

As part of the pre- and perioperative work-up, sophisticated imaging procedures, such as X-ray, ultrasound, CT-imaging, nuclear imaging (Positron Emission Tomography [PET], Single-Photon Emission Tomography [SPECT]) and magnetic resonance imaging (MRI) play a major role in modern surgery. Medical imaging delivers fundamental anatomical and biological information and plays a major role to enhance pre-operative planning and improve surgical image-guided interventions. Those imaging modalities have major influence on medical decision making, but they lack the necessary resolution and specificity to detect (pre)malignant and inflammatory tissue, especially during surgery. As it is well recognized that early detection of disease is essential to improve surgical outcome and patient survival, technical improvements should aim for disease-specific imaging methods in the surgical pathway. Novel imaging modalities such as optical and optoacoustic imaging have the potential to improve the surgical decision making (pre, intra- and postoperative) in a variety of surgical treatable diseases. These modalities allow for non-radiative *real-time* visualization of biological processes involved in for example oncogenesis discriminating tumor tissue from benign tissue in the field of surgical oncology, inflammatory disease such as inflammatory bowel disease in abdominal surgery, infectious diseases such as implant/bone infections in traumatology/orthopedics and vascular (minimal) invasive procedures for cardiovascular disease. In this thesis, we describe the clinical translation of targeted and generic fluorescent imaging agents to improve tumor detection for intra-operative fluorescence imaging (FI) purposes in a variety of tumor types. Moreover, we evaluate the clinical translation of optoacoustic imaging to improve the detection of vulnerable atherosclerotic plaques in vascular disease.

Improving intra-operative tumor control

Surgical excision remains an important treatment modality in most solid tumor types. For adequate local tumor control, an adequate and efficient excision with a tumor-negative margin is necessary, as a too narrow margin (i.e. tumor tissue in or near the border of the surgical specimen), or missed tumor depositions after excision encounter a significant decrease in disease-free and overall survival. Moreover, too excessive surgical excision could have major impact on the standard neurovascular anatomy, decreasing functional outcome. A tumor-positive surgical margin creates subsequent morbidity and costs due to adjuvant treatment.¹⁻⁴ Unfortunately, the incidence of a tumor-positive surgical margin remains significant, ranging from 6-21% in the 10 most common solid cancers and did not decrease over the last few decades despite improvements in surgical techniques.⁵

A variety of tumor types are discussed in this thesis and the high incidences of tumor-positive margins in all mentioned tumor types define that adequate discrimination between tumor tissue and benign tissue with the naked eye and palpation remains difficult and varies significantly between surgeons. For each solid cancer type the positive margin rates differs. For instance, soft tissue sarcoma (STS) is a rare cancer type, accounting for less than 1% of all cancers diagnosed.⁶ STS is the collective name for a heterogenous group of mesenchymal tumors with approximately 50 subtypes. The incidence of tumor-positive margins, i.e. tumor within 1 mm of the surgical margin, occurs up to 34% of all STS cases, resulting in approximately 35% local recurrence rates.^{7,8} Recurrence in sarcomas leads either to revision surgery with impaired surgical outcomes due to neurovascular or muscular morbidity or the inability to perform surgery, with the necessity of adjuvant chemotherapy or a palliative setting in irresectable sarcomas with subsequent poor survival. In oral cavity cancer, the incidence of tumor-positive margins is up to 23% worldwide.⁹ In breast cancer, after breast conserving surgery an astonishing 25% of all patients undergo additional surgery due to a positive surgical margin (tumor on ink) or by doubt of a non-sufficient tumor-negative margin.^{10,11} In the same order, for colorectal and esophageal cancer, a circumferential tumor-positive margin of 17% is observed with different tumor stages.¹² In patients with peritoneal metastases from colorectal cancer (CRC) origin, mortality rates due to recurrence of disease decreases with 39% after complete cytoreduction.

Depending on tumor type, tumor localization and disease stage, an inadequate resection induces high local recurrence rates, decreased survival rates and the necessity for additional adjuvant chemotherapy, radiative treatment or targeted treatment. Tumor cells from invading growing tumors tend to spread into surrounding tissue and anatomical deformations after radiotherapy or previous surgery complicate visual detection, emphasizing the need for an intra-operative *real-time* imaging technique for surgical guidance. Furthermore, more specific cancer detection on peritoneal carcinomatosis of CRC might also stratify those patients who will not benefit from these very extensive and mutilating surgical procedures and only have a negative impact on their quality of life with a prognostic impact. Increased visualization is especially important considering the current increase of minimal invasive (laparoscopic or robotic) surgery, which lacks tactile feedback.

Gaining more detailed anatomical and biological information during surgery is currently performed by several standard of care techniques. Frozen section analysis is performed in multiple surgical procedures to assess margin status using histopathology. However, this procedure is not *real-time*, delays surgery for a maximum of 1 hour and provokes a risk of sampling error, but shows an accuracy ranging up to 98% in multiple studies.^{13,14} The use of large imaging modalities like magnetic resonance imaging (MRI) and computed tomography (CT) is not practical or cost-effective for real-time intra-operative guidance in most oncological procedures and only provides anatomical and not tumor-specific information to the surgeon,. Functional imaging modalities like MRI, Positive Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT) can provide real-time biological and molecular information, but have practical drawbacks and are very costly as well. Nonetheless, these so-called molecular imaging techniques have gained enormous interest in the past decades and generated major insights in different molecular

pathways in oncology, cardiovascular and inflammatory and infectious diseases.¹⁵ The use of radioactive labeled imaging agents in oncology using PET and SPECT allows visualization of tumor biomarkers and almost all hallmarks of cancer, which has supported further understanding of *in vivo* spatiotemporal tumor behavior and allow for effective patient selection to assess an effective therapeutic strategy.^{16,17} Obtaining similar biological and molecular information can also be implemented *real-time* during surgery, using a variety of biomarkers which are overexpressed in tumor tissue compared to non-cancerous tissue.

Visualizing carotid atherosclerosis

Atherosclerosis of coronary and peripheral arteries is a chronic and inflammatory disease characterized by extracellular lipid accumulation in the intimal layer of the arterial vessel wall.¹⁸ These atherosclerotic plaques cause luminal narrowing of the affected artery. Carotid stenosis or even occlusion is a major cause of ischemic strokes and subsequent significant morbidity. There is consensus that biological active plaques are more prone for rupture than stable plaques.^{18–20} Such plaques are denominated as vulnerable plaques and are characterized by a thin fibrous cap with a necrotic core and increased macrophage infiltration.^{18–20} Nowadays, only the degree of stenosis (>70%) is the standard to select symptomatic patients for surgical carotid endarterectomy (CEA), but most ischemic strokes do not correlate with stenosis but with plaque rupture.²¹ There is a clinical unmet need for novel molecular imaging techniques to improve the diagnostic regimen and more accurate selection of patients for CEA with symptomatic carotid stenosis.²² Therefore, the identification of the vulnerable plaque with its prementioned characteristics could improve the preoperative selection, also for non-symptomatic patients with carotid stenosis. Intra-plaque vascularization with immature and unstable new blood vessel formation (i.e. angiogenesis) due to the necrotic / apoptotic core with upregulation of Hypoxia Inducible Factor – 1 α (HIF-1 α), the main driver of angiogenesis and therefore overexpression of vascular endothelial growth factor-A (VEGF-A), is a key factor in vulnerable plaques causing plaque hemorrhage.²³ Bevacizumab-800CW, a widely used GMP-produced imaging agent for oncological surgery in the UMCG consisting of the therapeutic antibody bevacizumab (Avastin) against angiogenesis and conjugated to the near infrared (NIR) dye IRDye800CW, has the potential to identify these highly VEGF-A expressing and lipid-rich vulnerable plaques by nuclear, optical or optoacoustic targeted imaging. Earlier reports previously showed the uptake of ⁸⁹Zr-bevacizumab on PET imaging in patients with carotid atherosclerosis.²⁴ As lipids have a distinct absorption spectrum in the near-infrared (NIR), identification of biological vulnerability with NIR imaging techniques might be possible. The aim was to evaluate both VEGF-A targeted imaging as well as the use of intrinsic absorbers in the evaluation of vulnerable plaques by optoacoustic imaging *in vivo* and *ex vivo* as a future non-invasive imaging technique to assess the (un-)stable carotid plaque.

OPTICAL IMAGING MODALITIES

Fluorescence imaging

Optical molecular imaging is an innovative clinical technique, combining the use of low-dose (non-) targeted imaging agents and specialized cameras with an appropriate light source in the NIR window to detect disease phenotype. Fluorescence camera systems perform excitation of a fluorophore on a specific wavelength, which immediately emits light in a longer wavelength

creating a fluorescence signal which can be detected by the imaging system. Different commercially available fluorescence camera imaging systems operate in the near infrared light spectrum (650-900 nm). In the University Medical Center Groningen, The Netherlands, a wide-field near-infrared fluorescence imaging camera is broadly used during surgery.^{25,26} NIR imaging allows for a penetration depth with high resolution up to ~1 centimeter.²⁷ Different tissue related intrinsic factors such as scattering and absorption properties and external factors such as external ambient light, homogeneity of the excitation light within a field-of-view (FOV), autofluorescence, imaging angle and imaging distance can significantly influence imaging results and therefore interpretation of the data acquired. Moreover, internal tissue components like hemoglobin level and necrosis can influence visualization as a result of differences in absorption properties causing light attenuation. These differences of absorption and scattering spectra in tumor tissue make a standardized methodology in a variety of tumors difficult as tumor and surrounding tissue components alters in between surgical specimens and patients complicating quantification. FGS allows for high-resolution imaging on the subcellular level and a variety of clinical trials in oncology have been assessed in patients using fluorescence and tumor-specific imaging agents for tumor detection and margin assessment, together with the identification of vital structures like nerves and arteries during surgery.²⁸⁻³⁴ Fluorescence-guided imaging is able to transform standard surgery relatively easy to implement imaging techniques towards an innovative real-time workflow.

Optoacoustic imaging

Surgical disease detection during the diagnostics pathway is necessary to enhance presurgical decision making and to decrease the number to treat in multiple areas. Due to the above mentioned internal and external factors reliable optical imaging is limited to a centimeter in depth. Spatial resolution of optical imaging decreases already after a few micrometer of depth due to light scattering.³⁵ Optoacoustic imaging (OAI) or photoacoustic imaging (PAI) is an innovative imaging modality which has the potential to penetrate deeper into tissue and give both anatomical and biological information for disease diagnosis and treatment monitoring. Endo- and exogenous molecules absorb light at a specific wavelength, undergo thermoelastic expansion and produce optoacoustic waves, making deeper penetration depths possible for imaging and can be detected by piezoelectric detectors.³⁵ Highly complex reconstruction algorithms are used to reconstruct such optoacoustic images. Different endogenous chromophores like hemoglobin, deoxyhemoglobin, melanin, fat and imaging agents like indocyanine green (ICG), IRDye-800CW and specific optoacoustic imaging agents with an absorption spectrum in the near-infrared can be identified in (pre-)clinical early phase imaging studies. Multispectral Optoacoustic Tomography (MSOT) is a macroscopic imaging methodology which uses a high-pulsed ND:YAG laser which illuminates the tissue of interest with multiple wavelengths to identify endo- or exogenous chromophores. Optoacoustic imaging combined with ultrasonography, the so-called hybrid approach, allows researchers to collect simultaneously anatomical information and biological/molecular information. Indications for (pre-)clinical MSOT imaging are broad, with main interests in oncology, inflammatory diseases and (peripheral) vascular disease. Landmark papers using clinical MSOT imaging have described the feasibility of MSOT imaging in breast cancer, thyroid cancer, inflammatory bowel disease, melanoma and Duchenne's disease.³⁶⁻³⁹ Also, high-resolution

3D MSOT images of peripheral arterial disease and carotid arteries were obtained.⁴⁰ Nowadays, there has been increasing interest in designing exogenous disease-specific photoabsorbent contrast agents to visualize formerly invisible disease. The ideal optoacoustic contrast agent should exhibit a high molar extinction coefficient, a low quantum yield and, a characteristic absorption spectrum with sharp peaks, high absorption in the NIR, high photostability, and high photothermal conversion efficiency.⁴¹ However, the greatest challenge is to produce a safe and generic imaging agent for single imaging use. Consequently, as a step-up approach we used a clinical available imaging agent, bevacizumab-800CW, for introduction of optoacoustic imaging in our already established fluorescence imaging workflow.

Multimodal imaging

Combining, in one and the same setting in for instance the surgical theatre, fluorescence imaging with optoacoustic imaging could provide both anatomical and functional tissue information with an improvement in depth penetration, imaging resolution and potential more accurate quantification by using optoacoustics. Fluorescence imaging will remain its use foremost in more superficial imaging purposes such as detection of peritoneal carcinomatosis in colorectal cancer, ovarian cancer or gastric cancer, whereas optoacoustic has the potential to guide the surgeon to examine deeper seated structures like regional lymph nodes and their metastatic involvement. Using tumor-specific (optoacoustic and fluorescent) imaging agents in oncology, this dual modality approach enables for non-invasive localization of tumor tissue and improve tumor detection and margin localization during surgery, creating a true clinical concept of 'shedding light onto live molecular targets', already postulated by Weissleder and Ntziachristos almost two decades ago.^{27,42} Introducing optoacoustic imaging besides fluorescence epi-illumination in our standardized clinical optical imaging workflow is needed and will be discussed in this thesis.⁴³

OUTLINE OF THIS THESIS

Fluorescence-guided surgery

The first part of this thesis describes the implementation and standardization of fluorescence imaging agents using fluorescence-guided surgery to increase tumor visualization during surgery. With this method, we aim to decrease the number of tumor-positive margins, which eventually will decrease morbidity and mortality rates. **Chapter 2** of this thesis focusses on fluorescence-guided surgery in soft tissue sarcomas (STS), a heterogeneous group of tumors with high rates of tumor-positive margins and therefore local recurrence.^{8,44} Overexpression of VEGF-A in STS has been described and the use of GMP-produced bevacizumab-800CW showed already clinical feasibility for in- and ex vivo imaging in breast cancer and peritoneal carcinomatosis in phase I and II clinical studies.^{25,30,45,46} In **chapter 2**, we describe the feasibility, safety and optimal dose-finding of bevacizumab-800CW during in STS patients. Moreover, we suggest a new image analysis platform using maximum fluorescence intensities at the resection margin to guide the surgeon during clinical decision making.

As the field of fluorescence-guided surgery is emerging in an exponential fashion, there is a clear need for a generic imaging agent which can be used in a variety of solid tumors and imaging systems. ONM-100 is a pH-activatable imaging agent consisting of micelles and

quenched indocyanine green (ICG), the latter already clinically used since the early 1970's with a well-known pharmacologic and safety profile. The micelles dissociate in an acidic environment, such as is present in the microenvironment of solid tumors and considered one of the hallmarks of cancer. Dissociation of the micelles causes unquenching of ICG and thereby the possibility of activation of fluorescence.^{16,47} In **chapter 3**, first-in-human safety, pharmacokinetic and imaging feasibility data of ONM-100 in four tumor types are presented, showcasing the tumor-agnostic characteristics of ONM-100.

Head- and neck tumors have one of the highest rates of tumor-positive surgical margins in oncological surgery.⁵ We analyzed all head- and neck cancer specimens in-depth in **chapter 4**. We describe the use of an analytical method where we determine the Tumor-to-Background Ratio (TBR) on the excised surgical specimen. We use a standardized specimen-driven fluorescence imaging approach to increase the detection and eventually decrease the amount of tumor-positive and close surgical margins. Moreover, we discuss the potential clinical benefits (i.e. residual tumor visualization, identification of tumor metastasis) in a variety of patients.

In **chapter 5**, we discuss the potential of fluorescence-guided NIR minimal invasive surgery e.g. laparoscopy in patients with peritoneal carcinomatosis to adequately visualize peritoneal metastases (PM) of colorectal origin. Overexpression of VEGF-A in colorectal PM has been observed and as such would serve as the optimal target for imaging purposes.⁴⁸ Two clinical FGS trials during cytoreductive surgery in PM patients have been performed using bevacizumab-800CW and SGM-101 targeting carcino-embryonic antigen (target CEA).^{30,49} Those tumor-specific imaging agents could be used for adequate patient selection during diagnostic laparoscopy and during extensive cytoreductive surgery.

Optoacoustic imaging

The identification of the vulnerable plaque, as described earlier, has the potential to differentiate symptomatic and asymptomatic patients which would have the presence of a vulnerable plaque to undergo a carotid endarterectomy. Previously, our group demonstrated the possibility of *ex vivo* visualization of matrix metalloproteinases in atherosclerotic plaques and the feasibility of vascular imaging using optoacoustic imaging.^{50,51} In **chapter 6** we investigated the *ex vivo* feasibility of targeting the vulnerable plaque with bevacizumab-800CW in an incubation setting to target VEGF-A expression and therefore angiogenesis in vulnerable carotid plaques. Angiogenesis is regarded one of the key-factors in the distinction between a stable and a non-stable plaque. In **chapter 7**, we translated our preclinical findings to a realistic clinical setting, where we investigated whether intravenous administration of bevacizumab-800CW combined with optoacoustic imaging could be used for the non-invasive detection of neovascularization of the vulnerable plaque.

Finally, **chapter 8** summarizes all discoveries of this thesis. Future perspectives for both fluorescence-guided surgery and the improvement in optoacoustic imaging is given in the future perspective section of this thesis. **Chapter 9** contains the Dutch summary of this thesis.

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CHAPTER 2

Fluorescence-guided visualization of soft tissue sarcomas by targeting vascular endothelial growth factor-A: a phase 1 single-center clinical trial

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ABSTRACT

Resection of soft tissue sarcomas (STS) is accompanied by a high rate of tumor-positive surgical margins (14-34%), which potentially leads to decreased disease-free survival. Vascular endothelial growth factor-A (VEGF-A) is overexpressed in malignant tumors, including STS, and can be targeted with bevacizumab-800CW during fluorescence-guided surgery for real-time tumor detection. In this phase 1 clinical trial, we determined the feasibility, safety and optimal dose of bevacizumab-800CW for fluorescence-guided surgery (FGS) in STS for *in-* and *ex vivo* tumor detection.

Patients with a histopathologically diagnosis of STS were included. In the dose escalation phase, patients received bevacizumab-800CW intravenously 3 days prior to surgery (10, 25 and 50 mg, n=8). In the subsequent dose-expansion phase, 7 additional patients received bevacizumab-800CW at the optimal dose. Fluorescence images *in-* and *ex vivo* were obtained during all stages of standard of care. The optimal dose was determined by calculating *in-* and *ex vivo* Tumor-to-Background ratios (TBR) and correlating these results with histopathology.

Fifteen patients with STS completed this study. All tumors could be visualized during *in-* and *ex vivo* imaging. The optimal bevacizumab-800CW dose proved to be 10 mg with a median *in vivo* TBR of 2.0 (± 0.58) and an *ex vivo* TBR of 2.67 (± 1.6). All seven tumor-positive margins could be observed real-time after surgical resection.

FGS using 10 mg bevacizumab-800CW is feasible and safe for intra-operative imaging of STS, potentially allowing tumor detection and margin assessment during surgery. An additional follow-up phase II study is needed to confirm the diagnostic accuracy.

INTRODUCTION

Soft tissue sarcomas (STS) account for less than 1% of all cancers diagnosed, with an estimated incidence in the United States of approximately 16,000 diagnosed annually.¹ STS comprises a histopathologically heterogeneous group of mesenchymal tumors, consisting of approximately 50 subtypes, with challenging diagnosis and treatment pathways.² Surgical excision remains the cornerstone of therapy for localized primary STS, and is usually combined with neoadjuvant or adjuvant radiotherapy. The main goal of surgery is to resect a STS with sufficient margin, excising the tumor and sufficient surrounding normal tissue. A tumor-negative margin is an important prognostic factor for local recurrence and disease-free survival.^{3,4} A tumor-positive margin, which occurs in 14-34% of the cases, results in local recurrence rates of approximately 35%.⁵ By histology, a tumor-positive margin is defined by tumor extension in the inked resection margin. However, microscopic tumor extension within 1 mm of the surgical margin may also be considered as a tumor-positive margin, as both conditions are associated with increased risk of local recurrence. In case of a tumor-positive margin an additional re-resection of the surgical cavity has to be considered.⁵ However, a re-resection can be challenging because of complex anatomy in the surgical field as a result of postoperative fibrosis and the presence of neurovascular structures, which increases risk of morbidity. Therefore, in many cases, proper clinical decision-making during surgery is a clinical trade-off in which achieving a tumor-negative surgical margin and functional outcome must be weighed. It is currently difficult for the surgeon to assess margin status during surgery, emphasizing the need for an intra-operative real-time imaging technique. During STS surgery, a clinician should be able to visualize tumor cells in the rim of the resection margin, both *in-* and *ex vivo*. Fluorescence-guided surgery (FGS) is an optical imaging method that provides real-time tumor detection and can be used for margin assessment. In the past years, several studies have investigated the potential merits of FGS in different malignancies such as breast cancer, peritoneal metastasis, colon cancer, glioblastoma and head and neck cancer.⁶⁻¹¹ These studies revealed promising data on the potential clinical benefit of detecting residual disease for intra-operative decision-making. For STS, only preclinical studies and one proof-of-concept study in a single patient using ABY-029 focusing on biomarkers like epidermal growth factor receptor for FGS have been reported.^{12,13}

In STS, a patient-tailored surgical plan is designed, based on tumor location and histopathological tumor classification, using immunohistochemistry and molecular genetics, and tumor grading.¹⁴ High-grade STS with extra-compartmental invasive growth has relatively decreased overall survival and event-free survival, by which one strives for an adequate wide en-bloc resection.¹⁵ In particular, myxofibrosarcomas are associated with tumor-positive margins, which is due to its diffuse reticular growth in subcutaneous fat or muscle, whereas other STS, e.g. myxoid liposarcomas tend to have a pushing invasive border, by which they are easier to excise, with narrow but clear margins.¹⁶

Vascular endothelial growth factor-A (VEGF-A) is involved in angiogenesis and lymph angiogenesis and is overexpressed in many solid tumors.¹⁷ Overexpression of VEGF-A in STS has been reported.^{18–20} The therapeutic monoclonal antibody Bevacizumab (Avastin®) binds to VEGF-A. By conjugating bevacizumab to the organic fluorophore IRDye800CW, a tumor-specific tracer (bevacizumab-800CW) could be developed.²¹ In previous studies using this tracer, bevacizumab-800CW was shown to be safe for use in humans.^{9,22}

The aim of this FGS study was to determine the feasibility, safety and the optimal dose of bevacizumab-800CW for *in-* and *ex vivo* detection and margin assessment of STS, using a standardized fluorescence imaging workflow.^{22,23}

MATERIALS AND METHODS

This phase 1 single-center feasibility study was performed at the University Medical Center of Groningen. The study was approved by the Institutional Review Board (IRB) of the University Medical Center of Groningen (IRB number 2017/302) and conducted according to the principles of Helsinki (adapted version Fortaleza, Brazil, 2013) and the laws and regulations of the Netherlands. The trial was registered at www.clinicaltrials.gov (NCT03913806). All patients provided written informed consent before participation in the study.

Patients

Patients aged older than 18 years with histopathologically proven STS and appropriate imaging (CT/MRI), scheduled for surgical excision, were included. All patients had a World Health Organization performance score of 0–2. Patients with concurrent invasive malignancy were excluded. Other exclusion criteria were: medical or psychiatric conditions compromising the patient's ability to give informed consent, pregnant or lactating women, a history of infusion reactions to bevacizumab, inadequately controlled hypertension or a history of myocardial infarction, transient ischemic attack, cerebrovascular accidents, pulmonary embolism, uncontrolled chronic hepatic failure or unstable angina pectoris six months prior to inclusion. All patients were discussed in the multidisciplinary sarcoma team meeting prior to surgery.

Study design

The primary objective of the study was to determine the feasibility of bevacizumab-800CW in STS for *in vivo* and *ex vivo* tumor detection. Secondary objectives were to identify the optimal dose for visualization of STS tissue and to obtain information on safety aspects of the tracer in STS patients. A classical 3 x 3 dose-finding study design was used, consisting of two parts. In the dose-escalation phase (part I) three dose cohorts were tested. Three subjects per cohort received intravenously administered flat-doses of respectively 10 mg, 25 mg and 50 mg three days before surgery. In the dose-expansion phase (part II), the optimal dosing group was expanded up to 10 subjects. The optimal dose group was chosen based on *in-* and *ex vivo* tumor-to-background ratio (TBR). The study design was adapted after the conclusion of part I to exclude patients who underwent neoadjuvant treatment in the dose-expansion phase.

Safety assessment

Vital signs were measured before, directly after and 1 hour after tracer administration. Follow-up was performed up to two weeks after tracer administration to assess adverse events which were scored according to the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) version 4.0.

Bevacizumab-800CW production

Bevacizumab-800CW was produced in the Good Manufacturing Practice (GMP) facility of the Department of Clinical Pharmacy and Pharmacology at the University Medical Center of Groningen as reported previously.²¹

Intra-operative fluorescence imaging

Fluorescence imaging of the tumor during surgery was performed with the SurgVision Explorer Air as described earlier.²² Once excision of the tumor was completed, fluorescence imaging of all resection planes of the surgical specimen was performed immediately (i.e. < 5 minutes) after excision with the SurgVision Explorer Air coupled to a closed-field imaging box (i.e. the Vault, SurgVision BV, Groningen, The Netherlands) and within 1 hour after excision with the PEARL Trilogy Imaging system (LI-COR BioSciences, Lincoln, NE, USA). Image acquisitions settings for Explorer Air and Pearl Trilogy were as described previously by our group for both *in-* and *ex vivo* imaging.²² No surgical decisions were made based on fluorescence imaging.

Fluorescence-guided pathology

The fresh surgical specimen was inked and serially sliced following standard protocols for pathological specimen handling. Fluorescence images of both sides of the tissue slices were obtained using the PEARL Trilogy. Next, tissue slices were formalin fixed for 1-4 days and imaged with the PEARL Trilogy before embedding (Figure 1). A pathologist, blinded for fluorescence, selected clinically relevant regions for further embedding in formalin-fixed and paraffin embedded (FFPE) blocks. Based on fluorescence imaging, additional regions of interest were selected which were also embedded in additional FFPE blocks. A standard fluorescence-guided workflow was used to cross-correlate the fluorescent tissue slices to final histopathology.²² Hematoxylin and eosin (H/E) stained slides were reviewed by a pathologist blinded for the results of fluorescence imaging. The complete workflow of the study is depicted in Figure 1.

Figure 1 | Standardized fluorescence imaging workflow

Patients received bevacizumab-800CW intravenously three days prior to surgery. During surgery, fluorescence imaging of the tumor *in vivo* was performed using SurgVision Explorer Air. Directly after excision, imaging of the whole surgical specimen was performed using SurgVision Explorer Air and PEARL Trilogy. After serial slicing performed by the pathologist, fluorescence imaging of all steps of histopathological processing was performed and cross-correlation with standard of care H/E slides was done.

Fluorescence quantification

The *in vivo* TBR was calculated as mean fluorescence intensity (MFI) of the tumor / MFI of the background. The tumor was delineated on macroscopic visualization. The background MFI was calculated on all non-tumor tissue surrounding the tumor in the surgical field. Mean Fluorescence Intensities (MFI, arbitrary units) were calculated on SurgVision Explorer Air images (exposure time 25 or 50 ms, gain 10 to 100). For the whole specimen imaging immediately after excision the maximum fluorescence intensities (MaxFI) were calculated on the whole resection margin to correlate signal intensity to margin depth.

Ex vivo TBRs were calculated on fresh serially sliced tissue slices. The tumor and the surrounding non-tumor tissue were precisely delineated on standard H/E histopathology by a pathologist blinded for fluorescence. An overlay with fluorescent tissue slices was made based on anatomical landmarks. Afterwards, an *ex vivo* TBR was calculated as mean fluorescence intensity (MFI) of the tumor / MFI of the background. The background MFI was calculated on all non-tumor tissue for every tissue slice.

Statistics

Descriptive statistics were reported as means with standard deviation (SD) in case of normal distribution, whereas median with interquartile range was used in case of a skewed distribution. Fluorescence signals in tumor and normal tissue were compared using the Mann-Whitney test. A P-value of <0.05 was considered statistically significant. For descriptive statistics SPSS (version 23.0; SPSS Inc, Armonk, NY) was used.

RESULTS

Between April and December 2019, fifteen patients with seven different histopathological subtypes of STS, including 7 myxofibrosarcomas, were enrolled in this dose escalation trial. Patient, safety and tumor characteristics are depicted in Table 1 and Supplemental Table 1. Bevacizumab-800CW was administered three days prior to surgery to all patients. No tracer related (serious) adverse events were observed (Table S1).

Phase 1 – dose escalation

A total of eight patients were included in the dose escalation phase (10 mg N=3, 25 mg N=3 and 50 mg N=2). All tumors were adequately visualized *in vivo* regardless of dose (10 mg: TBR 2.0 ± 0.58 ; 25 mg: TBR 2.5 ± 0.32 ; and 50 mg: TBR 2.0 ± 0.10) (Figure 2). After excision, tumor tissue was adequately visualized on the excised specimen (10 mg: TBR 2.0 ± 1.1 ; 25 mg TBR 2.5 ± 0.31). No reliable *ex vivo* TBR of 50 mg patients could be calculated due to lack of non-tumor background tissue. Since no increase in *in vivo* TBR was obtained for the 50 mg cohort after two patients, only two patients were enrolled in this cohort. Based on no statistical differences between the *in vivo* 10 and 25 mg cohort, ($p=0.18$), the 10 mg was considered to be the optimal dosing group and was expanded with 7 additional patients.

Table 1 | Clinical and pathological characteristics

	All patients (N = 15)
Patient characteristics	
Age at surgery (years), mean (range)	66 (34-84)
Sex: males, n (%)	8 (53)
Weight (kg), mean (range)	82 (64-103)
Height (cm), mean (range)	174 (161-189)
Dosing groups, n (%)	
10mg	10 (67)
25mg	3 (20)
50mg	2 (13)
Tracer related adverse events, n (%)	0 (0)
Tumor type primary tumor, n (%)	
Myxofibrosarcoma	7 (47)
High grade	6 (86)
Mixed type	1 (14)
Liposarcoma	3 (20)
Synovial sarcoma	2 (13)
Leiomyosarcoma	1 (7)
Angiosarcoma	1 (7)
Undifferentiated	1 (7)
pleomorphic sarcoma	
Tumor diameter (mm), mean (range)	112 (18-320)
Localization tumor, n (%)	
Arms	4 (27)
Legs	4 (27)
Intraabdominal	4 (27)
Hip/flank	2 (13)
Breast	1 (7)

Data are given in numbers with percentages (%) or means with range

Figure 2 | In vivo visualization of soft-tissue sarcomas

Intra-operative imaging was performed with the SurgVision Explorer Air. Fluorescence and white light images of an intra-abdominal leiomyosarcoma in vivo using 10 mg bevacizumab-800CW and a synovial sarcoma using 25 mg bevacizumab-800CW were obtained (A). Median in vivo Tumor-to-Background Ratio (TBR) for all patients in the dose escalation phase (n=8) were calculated. Median in vivo TBRs are calculated per dosing group (B).

Ex vivo fluorescence and quantification on tissue slices

To quantify fluorescence, quantification on fresh tissue slices was performed. All seven histopathological STS subtypes could be visualized *ex vivo*, displaying a sharp, clearly delineated fluorescent signal in all tissue slices containing tumor (Figure 3). Significantly higher fluorescence signals were seen in tumor tissue compared to non-tumor tissue in the 10 mg group (Figure 3, n = 22, $P < 0.001$, median MFI of 0.013 for tumor tissue and median MFI of 0.004 for non-tumor tissue). The median *ex vivo* TBRs of the 10 mg and 25 mg dose cohort were 2.67 ± 1.6 (n = 9, range 1.5-6.5) and 4.6 ± 0.31 (n=2, range 4.5-4.7) respectively. In the STS series, all myxofibrosarcomas (n=7) could be visualized using fluorescence during *ex vivo* imaging (Supplemental Figure 1). Due to the imaging resolution of the PEARL Trilogy, the border zone with strands of diffusely infiltrating tumor cells could not be individually identified with visual fluorescence inspection. In this study, we found that more solid and cellular tumor areas are easily identifiable.

In the tissue slices, a false-positive fluorescent signal could be observed in areas with a high macrophage content. We suspect this occurred due to an inflammatory response after tumor necrosis induced by neoadjuvant radiotherapy (Supplemental Figure 1, a-c, m-o). For this reason, patients who received neoadjuvant radiotherapy were not eligible to participate during the dose expansion phase, to minimize false-positive non-tumor tissue signal.

Figure 3 | Ex vivo visualization of soft tissue sarcomas
Leiomyosarcoma and myxofibrosarcoma with whole specimen image of a close surgical margin on the basal resection margin (0.6cm) and a close surgical margin on the caudal resection margin (0.6cm). Fluorescence signal on tissue slice with the tumor delineated with a dashed line and a close surgical margin on the basal resection margin and caudal resection margin. The tumors are delineated with a solid black line on standard H/E staining (A). Sub-analysis in the 10 mg group showed a significantly higher mean fluorescence intensity (MFI) in all tumor tissue compared to non-tumor tissue ($n = 22$, $P < 0.001$) (k) and all ex vivo TBRs are presented for all patients in the 10 mg group, $n = 8$, no data available for FLASH03 (false-positive signal) and FLASH13 (angiosarcoma, no reliable calculation possible) (B).

Fluorescence margin assessment

After histopathological assessment, seven patients were diagnosed with a tumor-positive surgical margin (47%), containing three high-grade myxofibrosarcomas, two high-grade undifferentiated sarcomas, and one synovial sarcoma. In one retroperitoneal well-differentiated and focally dedifferentiated liposarcoma the margin was considered positive due to size of the disease and limitations in surgical excision but was not included in our fluorescence analysis. All tumor-positive surgical margins were detected using fluorescence imaging of the resected specimen (7/7). Three patients had a histological proven tumor-negative surgical margin (20%), of which fluorescence predicted 1/3 correct (33%). One margin was false-positive due to neoadjuvant

treatment while the other margin was false-positive due to a highly fluorescent cyst. Five patients had a histological proven close surgical margin (33%) ranging from 0.6 mm to 1.4 mm, of which one was false-positive for fluorescence due to neoadjuvant treatment and therefore excluded in our analysis (n=4). Quantification of MaxFI on whole specimen imaging with a close surgical margin (0.6 - 1.4 mm) showed a non-significant trend in MaxFI on resection margins with the closest surgical margin (Figure 3). For all patients (n = 4, 13 resection margins evaluated) with a close surgical margin (range from 0.6 mm – 1.4 mm) the surgical side with the closest surgical margin had the highest maximum fluorescence intensity, suggesting a relation between maximum fluorescence intensity and margin depth (Figure 4).

Figure 4 | Ex vivo whole specimen imaging directly after excision

Immediately after surgical excision (< 5 minutes) standardized fluorescence images of all resection planes of the surgical specimen were obtained. In this myxofibrosarcoma, the caudal resection margin showed a high maximum fluorescence intensity (MaxFI=901) which correlated to a close surgical margin of 6mm, whilst the cranial resection margin showed a lower MaxFI of 612 correlating with a deeper resection margin of 13mm. Maximum fluorescence intensity signals were calculated on all close resection margins from four patients. 1 is the closest surgical margin as described by standard histopathology and 4 is the widest surgical margin. Per patient analysis for patient FLASH12 is presented.

DISCUSSION

In this pilot study, we have shown that fluorescence-guided surgery using bevacizumab-800CW is feasible and safe in a variety of histological subtypes of soft tissue sarcomas using both *in-* and *ex vivo* fluorescence imaging. 10 mg bevacizumab-800CW proved to be safe and sufficient for adequate *in-* and *ex vivo* imaging, whereas a dose of 25 mg or 50 mg did not significantly improve fluorescence visualization. Moreover, we show that FGS has great potential value for tumor detection and margin assessment during STS surgery. This is evident after studying depth assessment for close surgical margins using *ex vivo* fluorescence-guided specimen imaging immediately after excision, as shown in previous FGS studies.^{22,24} We believe that this technique has the potential to optimize surgical quality and decrease the amount of tumor-positive surgical margins.

Tumor histotype and grade as well as anatomical location of STS are important determinants of local recurrence. Ideally, FGS should reflect tumor infiltration patterns (diffuse vs. pushing infiltrative borders) during surgery. Our data on fluorescence visualization of myxofibrosarcomas confirm that this tumor is accompanied by frequent positive surgical margins due to diffuse, reticular invasive growth in surrounding soft tissue. Despite the fact that the solid cellular tumor areas could be easily identified, future studies should determine whether FGS can enhance intra-operative margin assessment in diffusely infiltrative STS.

In our previous studies in breast cancer we confirmed the co-localization of bevacizumab-800 with VEGF-A IHC. In 90% of all patients there was adjacent/complete overlap of bevacizumab-800CW and VEGF-A immunohistochemistry staining, which also could be observed in immunohistochemistry analysis in STS tissue (Supplemental Figure 2).¹⁰ As VEGF-A staining is heterogenous in different STS subtypes and would only be reliable if executed thoroughly analyzing the whole surgical specimen, we did not include a full IHC analysis in this study. Nevertheless, VEGF-A expression in myxofibrosarcomas has recently been observed in 93% as a suitable marker for FGS and VEGF-A expression significantly correlated with clinical variables like high histological grade and distant metastasis.^{25,26} Our imaging results, showing specific fluorescence activation in areas containing soft tissue tumor as assessed by H/E staining confirm these preclinical results and are in accordance with earlier clinical reported data. Tumor necrosis, frequently observed in high-grade STS, may be induced by neoadjuvant radiotherapy. In general, tumor necrosis is a significant predictor of relapse-free survival and overall survival in STS patients.²⁷ Bevacizumab-800CW is not adequately visualized in necrotic areas, as no adequate penetration and specific binding could be obtained. We observed false-positive fluorescent signals in areas with high macrophage content and reactive fibrosis related to the inflammatory response after neoadjuvant radiotherapy. Therefore, FGS with bevacizumab-800CW for real-time margin assessment seems to be most suitable during primary surgery with the use of radiotherapy in an adjuvant setting. In a phase II study, a cohort with a significant amount of neoadjuvant treated patients should be included to compare the results of fluorescence-guided surgery in patients with soft tissue sarcomas undergoing neoadjuvant or adjuvant treatment.

As previously described in head- and neck specimens, quantification of all resection planes of the whole specimen directly after surgical excision using a fluorescence signal surface map, may identify margin depth with fluorescence intensity peaks. The margin segment with the highest fluorescence intensity was termed the sentinel margin-the location where the closest margin is mostly likely to be located.²⁸ Although we could correlate the maximum fluorescence intensity to a close surgical margin, our study was not powered to determine the invasion depth on a microscopic level. Currently, it is challenging to determine sentinel margins in sarcoma surgery as fluorescence imaging of especially the deep resection margins is not feasible using the current available large intra-operative imaging devices. Sarcomas are usually in tight anatomical spaces requiring challenging positioning of intra-operative devices. Furthermore, the variation in imaging acquisition settings and the absence of standardization may result in a lack of quality control. *Ex vivo* fluorescence imaging with so-called closed-field cameras enables better control of image acquisition parameters and removes interference of ambient light. Our *ex vivo* analysis can be performed within 5-10 minutes after surgical excision, as *ex vivo* imaging enables a standardized and reproducible image analysis platform to guide surgical decision making. Standardizing fluorescence imaging could minimize signal inhomogeneity that can lead to erroneous tumor delineation. In a future scenario, on-site examination in the surgical theatre by a pathologist equipped with a fluorescence imaging system may guide the surgeon in adapting surgical treatment decisions. Based on intra-operative imaging findings and the outcome of fluorescence-guided pathology, this may potentially prevent both under- and over-treatment and diminish surgical reinterventions at a later stage.

CONCLUSION

To conclude, this study is a first proof-of-concept that FGS using a flat-dose of 10 mg bevacizumab-800CW is feasible in a variety of STS subtypes. Moreover, the potential value of fluorescence imaging during *ex vivo* pathology assessment was evident. FGS appears to be most valuable in patients undergoing primary surgery without neoadjuvant treatment. A subsequent phase-II trial will be performed in our institute to determine the sensitivity and specificity of FGS in patients treated with primary surgery with curative intent.

SUPPLEMENTAL DATA

Supplemental Figure 1 | Fluorescence of myxofibrosarcomas

Representative ex vivo images of six myxofibrosarcomas imaged with PEARL Trilogy in this study. The last myxofibrosarcoma is presented in Figure 3 panel f-j. The tumor is delineated on H&E staining with a black line, the white and black dotted lines represent the tumor on fluorescence and white light images. In two patients, a false-positive signal is observed possibly due to tumor necrosis and macrophage infiltration due to neoadjuvant treatment (a-c, m-o).

Supplemental Figure 2 | VEGF-A staining

Microscopic localization of bevacizumab-800CW in a myxofibrosarcoma. The T represents tumor tissue and the Asterix represents muscle tissue. High intensity of Vascular Endothelial Growth Factor-A is observed in tumor tissue.

Supplemental Table 1 | Safety data

Dose Group	(S)AE	CTCAE	Description	Events	Moment	Relation to tracer
1	AE	1	Postoperative pain	6x	Postoperative	Not related
2	AE	1	Hematochezia	1x	*	#
3	AE	2	Opioid intoxication	1x	*	#
4	AE	1	Postoperative pain	2x	*	#
5	AE	2	Mild pancreatic injury after surgery	1x	*	#
6	AE	1	Postoperative pain	2x	*	#
7	AE	1	Hot flashes	1x	*	#
8	AE	1	Fever	1x	*	#
30	AE	1	Disorientation	1x	*	#

*All post-operative. #All not related. Abbreviations: (S)AE, (serious) adverse event; CTCAE, common terminology criteria for adverse events

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CHAPTER 3

Exploiting metabolic acidosis in solid cancers using a tumor-agnostic pH-activatable nanoprobe for fluorescence-guided surgery

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ABSTRACT

Cancer cell metabolism leads to a uniquely acidic microenvironment in solid tumors but exploiting the labile extracellular pH differences between cancer and normal tissues for clinical use has been challenging. Here we describe the clinical translation of ONM-100, a nanoparticle-based fluorescent imaging agent. This comprises of an ultra-pH sensitive amphiphilic polymer, conjugated with indocyanine green, which rapidly and irreversibly dissociates to fluoresce in the acidic extracellular tumor microenvironment due to the mechanism of nanoscale macromolecular cooperativity. Primary outcomes were safety, pharmacokinetics and imaging feasibility of ONM-100. Secondary outcomes were to determine a range of safe doses of ONM-100 for intra-operative imaging using commonly used fluorescence camera systems. In this study (Netherlands National Trial Register #7085), we report that ONM-100 was well tolerated and four solid tumor types could be visualized both *in-* and *ex vivo* in thirty subjects. ONM-100 enables detection of tumor-positive resection margins in 9/9 subjects and four additional otherwise missed occult lesions. Consequently, this pH-activatable optical imaging agent may be clinically beneficial in differentiating previously unexploitable narrow physiologic differences.

INTRODUCTION

Cancer specific fluorescence-guided imaging has traditionally involved fluorophore-labeled small molecules, antibodies, nanobodies, peptides or nanoparticles against cell surface receptors.^{1–9} Despite promising results, such strategies often lack broad tumor applicability due to the diversity of oncogenotypes and phenotypes.^{10,11} In contrast, extracellular cancer acidosis – a ubiquitous consequence of cell proliferation and growth in cancer – could serve as a generic target in a variety of solid tumors.^{12,13} While virtually all solid cancers demonstrate low extracellular pH relative to the tightly regulated pH of the normal extracellular compartment, the pH is spatiotemporally variable. The variability in tumor pH is affected by tumor intrinsic factors, such as aerobic and anaerobic glycolysis, hypoxia, angiogenesis and perfusion, that interact in unpredictable ways with different areas of a given cancer resulting in a pH between 5.8–7.4 at different times.^{14,15}

To exploit the dysregulated tumor milieu, a series of tunable pH-sensitive amphiphilic polymers have been developed that generate a fluorescent output in response to a reduction in pH.¹⁶ In an aqueous solution, the polymers self-assemble into nanometer sized micelles. Fluorophores, such as indocyanine green (ICG), sequester within the hydrophobic segments of the micellar core leading to homoFRET fluorescence quenching.¹⁷ Cooperative dissociation of the micelles at a tunable pH threshold releases the individual unimers, thereby unquenching the fluorescent dye (Figure 1). The discrete cooperative response of the polymers to pH is a unique nanoscale phenomenon with two distinguished characteristics for pH sensing.¹⁸ First, the cooperativity, with a Hill's coefficient as high as 51, makes fluorescence activation a thresholded maximal event: fluorescence is either completely off or completely on above and below a sharply demarcated pH point. Second, the activation is irreversible due to the capture of dissociated unimers by the proteins in the tumor microenvironment which prevent reformation of the micelles that would quench the fluorescence. When progressive fluorescence activation in zones of acidosis is integrated over time, a stable, discrete fluorescent labeling of tumors is achieved.¹⁹

Human patients vary widely with respect to body habitus and underlying metabolic and nutritional status, while their cancers vary by type, size and mutational status.²⁰ These extrinsic and intrinsic tumor variables compound accurate tumor pH detection. Despite Otto Warburg first describing deregulated cancer metabolism over a century ago, it has not been utilized clinically. We therefore hypothesized that the nanoscale cooperativity of the pH responsive polymers could be leveraged for fluorescence-guided tumor identification and that the irreversible binary activation could provide novel information not obtainable through routine surgical standards of care.

In this clinical study, ONM-100, an intravenously administered imaging agent based on pH sensitive polymers, was investigated in human patients. The results presented below demonstrate ONM-100's ability to detect, otherwise missed, tumor-positive surgical margins and occult disease. All four of the investigated solid tumor types were visualized by tumor agnostic fluorescence. The potential of ONM-100 in clinical decision making during and post-surgery needs to be confirmed by an additional Phase II clinical study.

Figure 1 | Mechanism of action

A tumor microenvironment turns acidic when the tissue transforms from pre-malignant cells into invasive cancer. The ONM-100 nanoparticles extravasate due to the enhanced permeability of the tumor vasculature and are then retained due to the poor lymph drainage in the tumor tissue. This leads to ONM-100 accumulation in the acidic extracellular matrix causing the pH activated fluorescence to switch from the "off" (green) to the "on" (red) state. Abbreviations: pH_e : Extracellular pH, pH_t : threshold pH.

RESULTS

Summary of safety and pharmacokinetic evaluations of ONM-100

The primary outcomes of this Phase 1 study were the safety, pharmacokinetics and imaging feasibility of a single intravenous dose of ONM-100 administered to patients with solid cancer 24±8 hours prior to undergoing standard surgery. The study procedures are shown in Figure 2. Thirty subjects with four different solid tumor types (head and neck squamous cell carcinoma [HNSCC], $n=13$, breast cancer [BC], $n=11$, esophageal cancer [EC], $n=3$, and colorectal cancer [CRC], $n=3$) were enrolled.

Figure 2 | Study design

Intravenous administration of ONM-100 was performed approximately 24h ($\pm 8h$) prior to surgery. Ten days of safety assessments (laboratory, pharmacokinetics, ECGs) followed, adverse events were monitored up to day 17 (a); During surgery, intraoperative images were obtained prior to incision and after excision of the surgical cavity; (b) Immediately after excision the specimen was imaged for the presence of a tumor-positive surgical margin; (c) Fluorescence images were obtained during all the standard pathology processing phases (d,e); and the H/E slices were correlated with the standard histopathology slices (f-h). Abbreviations: i.v.: intravenous; ECG: electrocardiogram; H/E: Hematoxylin Eosin; SOC: standard of care.

Eight subjects with HNSCC and seven with BC were included in the dose-escalating Phase 1a study (secondary outcome). To evaluate the tumor agnostic imaging feasibility further with ONM-100, 15 additional subjects with four different tumor types (HNSCC, BC, EC, or CRC) were dosed in Phase 1b with an optimal dose determined in Phase 1a (Table 1 and Supplementary Table Table 1). All 30 subjects completed the study. ONM-100 was well-tolerated by all 30 subjects with no dose limiting toxicities or drug related Serious Adverse Events (SAEs). Four possible drug related Common Terminology Criteria for Adverse Events (CTCAE) grade 1 adverse events (AEs) were observed in the 0.3 mg per kg cohort (Table 2). General blood counts, determined up to day 10, showed no imaging agent related aberrations. The plasma exposure (C10m and AUC 0-24h) of ONM-100 was linearly correlated with the dose, with an R2 of 0.95 and 0.98, respectively (Supplementary Figure 1). The mean terminal-phase half-life in the 1.2 mg per kg cohort was 44.5 with a standard deviation of 15.8 hours. No differences in pharmacokinetics were observed among the different tumor types (Supplementary Figure 2).

Table 1 | Study characteristics

Surgical resection margins are displayed according to the US Guidelines per tumor type. Adverse events are defined as possible imaging agent related adverse events. *Two primary tumors from the same subject. Abbreviations: BC: Breast Cancer; HNSCC: Head and neck squamous cell cancer; EC: Esophageal Cancer; CRC: Colorectal Cancer; SD: Standard Deviation; Tx: Treatment; NST: No special type; AE: Adverse Event. Source data are provided as a Source Data file

	All N=30	0.1mg/kg N=3	0.3mg/kg N=3	0.5mg/kg N=3	0.8mg/kg N=3	1.2mg/kg N=18
Patient characteristics						
Age, mean (range)	63 (35-85)	68 (46-80)	53 (35-63)	57 (50-69)	61 (53-78)	65 (34-85)
Males, number (%)	8 (27%)	1 (33%)	0 (0%)	1 (33%)	0 (0%)	6 (33%)
Weight, mean (range)	80 (47-113)	62 (53-69)	89 (71-113)	88 (52-119)	87 (73-94)	79 (47-110)
Safety data						
AE Grade I	0	0	3	0	0	0
AE > Grade I	0	0	0	0	0	0
Tumor type						
<i>BC</i>						
Invasive carcinoma	7	0	2	1	2	2
<i>NST</i>						
Carcinoma with medullary characteristics	2	0	1	0	0	1*
Mucinous arcinoma	1	0	0	0	0	1
Lobular carcinoma	1	0	0	0	0	1
Invasive micropapillary carcinoma	1	0	0	0	0	1*
<i>HNC</i>						
HNSCC	13	3	0	2	1	7
<i>EC</i>						
Intestinal adenocarcinoma	1	0	0	0	0	1
Mixed adenoneuroendocrine carcinoma	1	0	0	0	0	1
No viable tumor (after Neoadjuvant Tx)	1	0	0	0	0	1
<i>CRC</i>						
Intestinal adenocarcinoma	3	0	0	0	0	3
Tumor size						
Max diameter, mean ($\pm$ SD)	2.4 ($\pm$ 1.6)	1.9($\pm$ 0.4)	2.2 ($\pm$ 0.8)	3.6 ($\pm$ 1.0)	3.6 ($\pm$ 2.2)	2.1 ($\pm$ 1.6)
Surgical resection margins						
Tumor-negative	18	11	2	2	2	1
Tumor-positive	9	4	1	1	1	2

Table 2 | Adverse events

Adverse events that occurred during the course of the study which were possibly related to imaging agent administration and scored according to the Common Terminology Criteria for Adverse Events (CTCAE) v4.03: June 14, 2010. Abbreviations: BC: Breast Cancer.

Tumor type	Dose cohort	Adverse Event	Grade	Intervention	Resolved
BC	0.3mg per kg	Headache	I	No	Yes
BC	0.3mg per kg	Pain left cheek	I	No	Yes
BC	0.3mg per kg	Flush	I	No	Yes
BC	0.3mg per kg	Dizziness	I	No	Yes

Summary of fluorescence imaging results

Viable tumors were confirmed in a total of 29 of the 30 enrolled subjects by histology. In each case, a sharp demarcated fluorescent signal was visible irrespective of the tumor type or dose (Figure 3) based on the ex vivo standard fluorescence workflow analysis described previously (Figure 2).²¹ We confirmed the tumor specific activation of ONM-100 ex vivo by administering it topically on tissue sections of a freshly frozen HNSCC specimen (Supplementary Figure 3). Moreover, no fluorescence was visible in the blood samples prior to acidification (Supplementary Figure 4).

Figure 3 | Fluorescence images of different tumor tissue slices

Head and Neck Squamous Cell Cancer of the tongue (a-f); Breast Cancer (g-l); Esophageal Cancer (m-r); Colorectal Cancer (s-x). The tumor is delineated as a solid black line in the H/E slices (c, i, o, u). The Mean Fluorescence Intensity (MFI) of the tumor tissue and the non-tumor tissue slices, per tumor type, is depicted (y). The dots represent the MFI of single tissue slices (≈ 3 per subject) from the 1.2 mg per kg cohort. HNSCC, 7 subjects, $P < 0.0001$, BC, 5 subjects, $P = 0.0001$, EC, 3 subjects, $P = 0.0010$, Wilcoxon test, two-sided. CRC, 3 subjects, no statistics performed due to the availability of only three data points. Abbreviations: HNSCC: Head and Neck Squamous Cell Cancer; BC: Breast Cancer; EC: Esophageal Cancer; CRC: Colorectal Cancer; H/E: Hematoxylin Eosin; ***: $P \leq 0.001$; ****: $P \leq 0.0001$. Source data are provided as a Source Data file

Thirteen HNSCC, the five superficially seated BC tumors and two CRC tumors were visualized in-situ in real time by ONM-100 fluorescence during surgery. All the tumor-positive surgical margins (9 out of 9) that were undetected during standard of care (SOC) surgery were visualized during intraoperative fluorescence imaging and correlated with the final histopathological assessment, yielding 100% sensitivity and no false negatives (Table 3 & Figure 4). In addition, ONM-100 fluorescence was detected in five additional occult lesions (in 1 HNSCC and 4 BC subjects) otherwise missed by the SOC surgery or pathological analysis. The histologically proven peritoneal metastases (PM) of the two CRC subjects were fluorescent both in- and ex vivo whereas their non- malignant excised lesions did not fluoresce (Supplementary Figures 5 and 6 and Supplementary Movie 1-4).

Figure 4 | Fluorescence-guided assessment of surgical margins

Representative example of a head and neck squamous cell carcinoma of the tongue from a subject with a negative surgical margin. In- and ex vivo visualization of fluorescence in the tumor (a, c, g, i) with no fluorescent signal in the surgical cavity or at the surgical resection margin (b, h, d, j). Correlation of fluorescent signals on a tissue slice with the histology (e, k, f) with a tumor negative surgical margin of 6.4mm. Representative example of breast cancer surgery (i.e., a lumpectomy) with a tumor-positive surgical margin (l,m,n,o). Fluorescence is detected at the ventral surgical margin both in vivo and immediately after excision (r, s, t, u) which corresponds with the fluorescence localization on the tissue slice (p, v) and the final histopathology (q). The tumor is delineated as a solid black line on the H/E slices (f, q). Abbreviations: H/E: Hematoxylin Eosin; SOC: standard of care.

Table 3 | Contingency table for fluorescence-guided surgical margin assessment

Intraoperative assessment of the surgical margin during fluorescence-guided surgery was either done by intraoperative fluorescence imaging of the surgical cavity or fluorescence imaging of the excised specimen at the 'Back-Table'.

	Tumor-positive resection margin	Tumor-negative resection margin
Fluorescence positive	9	5
Fluorescence negative	0	10
Total	9	15

The tumor agnostic nature, diagnostic accuracy and potential clinical utility of ONM-100 are further described below. Three of the BC subjects (27%) had a tumor-positive surgical margin, all of which were detected by fluorescence imaging. Eight BC subjects had a proven histopathological tumor negative surgical margin of which six were assessed correctly by fluorescence imaging, corresponding to a sensitivity of 100% and a specificity of 75% for ONM-100 in this sample size (Supplementary Figure 7 and Supplementary Table 2). Of the two BC subjects with a false positive fluorescence imaging result, one subject had a histopathological tumor negative margin as defined by the ASTRO/SSO guidelines. However, it contained a ductal carcinoma in situ (DCIS), an entity with cancer cells within the wall of the ductuli and, according to international guidelines, might require additional surgery, underscoring the clinical utility of detecting this lesion. Hence, it can be debated if this represented a true false positive sample. In the other BC subjects with a false positive fluorescence imaging result, the fascia of the larger pectoral muscle showed a homogenous higher fluorescent signal, as also described previously. Regarding the HNSCC cohort, a total of six subjects (46%) had a histopathologically confirmed tumor-positive surgical margin, all of which were detected intraoperatively by fluorescence. A tumor-negative surgical margin was confirmed histopathologically in the remaining seven subjects (54%). Three false positive fluorescence margins were detected, which did not contain tumor on final histopathological examination (Supplementary Figure 5), resulting in a sensitivity of 100% and a specificity of 57% among the HNSCC subjects (Supplementary Figure 7 and Supplementary Table 1).

The ability to detect occult disease such as satellite metastases and second primary tumors, which are missed by SOC procedures, exemplifies the potential clinical utility of ONM-100. This was an ad hoc outcome parameter of the study. A satellite metastasis in one of the HNSCC subjects was undetected by SOC surgery but was detected in the surgical cavity by fluorescence imaging (Supplementary Figure 5). As discussed above, the DCIS was detected by ONM-100 fluorescence in the surgical cavity and back-table in a specimen of a BC subject and was confirmed by histopathology. Moreover, fluorescence imaging during histopathological processing detected three additional otherwise missed cancers in three BC subjects. Of these,

fluorescence imaging enabled the detection of additional satellite BC metastasis in the surgical specimens of two subjects and a second primary tumor lesion (triple negative BC) in the third subject (Supplementary Figure 5).

One deeper seated intraluminal rectal, two intraluminal esophageal and six deeper seated breast tumors could not be visualized intraoperatively due to the near-infrared (NIR) imaging technology. Notably, however, none of the intraluminal or deep-seated tumors had a tumor-positive margin on final pathology. Importantly, the fluorescence imaging procedure did not interfere with the SOC of any of the tested subjects and, generally, the surgical procedures were only prolonged by a maximum of 10 minutes.

Fluorescence quantification and Tumor to Background Ratio

Ex vivo workflow analysis, to further validate the intra-operative findings, showed that the tumor tissue of all the subjects with histopathologically proven viable tumor tissue showed a higher fluorescence signal intensity with sharp morphological delineations on the tissue slices compared to normal tissue, irrespective of tumor type and dose cohort (Figure 3, panel y and Supplementary Figure 6). The tumor tissue's mean fluorescence intensity (MFI) in tumor tissue increased with dose (Figure 5, panel a). In all cohorts, the tumor MFIs were significantly higher than that of non-tumor tissue (Figure 5). The median tumor-to-background ratio (TBR) of all the tissue slices ($n=97$ from 26 subjects) was 4.5 with an interquartile range (IQR) of 3.1. (Figure 5, panel a). The optimal dose for tumor detection and sensitivity according to the Phase 1b studies was 1.2 mg per kg (TBR 4.5, IQR 3.0) and the MFI of that dose group's ($n=15$) tumor tissue was significantly higher compared to normal tissue in each of the available tissue slices ($n=59$, $P<0.0001$, Wilcoxon test). A Receiver Operators Characteristics (ROC) curve analysis of these tissue slices showed an area under the curve (AUC) of 0.9875, $P<0.0001$ (Figure 5, panel g).

An *in vivo* TBR was calculated for the mucosal tumors (HNSCC) which were directly exposed to the surface; deeper seated tumors gave less reliable results due to overlaying tissue and differences in absorption and the scattering properties of the tissue. The median *in vivo* TBR of the HNSCC subjects in the 1.2 mg per kg group was 2.6 with an interquartile range of 1.4 (representative examples shown in Supplementary Figure 8).

Figure 5 | Dose independent Mean Fluorescence Intensity separation between tumor tissue and non-tumor tissue.

Tumor and non-tumor tissue Mean Fluorescence Intensities (MFI) from the: 0.1 mg per kg cohort, $P=0.0005$ (a); 0.3 mg per kg cohort, $P=0.0078$ (b); 0.5 mg per kg cohort, $P=0.0020$ (c); 0.8 mg per kg cohort, $P=0.0078$ (d); and 1.2 mg per kg cohort, $P<0.0001$, Wilcoxon test, two-sided. (e). The dots represent the MFI of single tissue slices. The Receiver Operator Characteristics Curve is based on the calculated MFI of the tumor and normal tissues from the 1.2 mg per kg dose-cohort, $P<0.0001$; Area Under the Curve 0.9875, $n=59$, with a confidence interval of 95% using Wilson/Brown Method (f). Abbreviations: ROC: Receiver Operators Curve; AUC: Area under the Curve; $**$: $P\leq 0.01$; $***$: $P\leq 0.001$; $****$: $P\leq 0.0001$. Source data are provided as a Source Data file.

DISCUSSION

The *in-* and *ex vivo* data from this fluorescence imaging study indicate that the low pH resulting from tumor acidosis can be exploited as a generic biomarker for cancer in patients with four different solid tumor types including head and neck squamous cell carcinoma, breast cancer, esophageal cancer and colorectal cancer. The pH-sensitive fluorescent imaging agent ONM-100 was well tolerated and was activated by tumor acidosis, delineating tumors from benign tissues. The initial clinical findings from thirty subjects provide information about occult cancer which would otherwise not be obtained by the standard of care (i.e., visual inspection and palpation alone), which was illustrated by the intraoperative detection of all the tumor-positive surgical margins (9 out of 9), a DCIS and a satellite cancer in a head and neck cancer subject as well as the *ex vivo* detection of three additional satellite lesions and second primaries in the pathology specimens.

This Phase I study is the first example of a systemically administered agent that displays nanoscale cooperativity which overcomes metabolic and phenotypic variability between different patients and tumors. Most importantly, and in contrast to recently studied fluorescence imaging agents, there was no overlap between tumor and background fluorescence for any given subject.^{1–6,8,22}

Two features of the reported data are unique. First, while tumor acidosis was first described almost 100 years ago, clinical use of this physiologic characteristic of cancer has been hampered by the spatiotemporal variability of pH in tumors and the wide array of factors that can impact tumor acidity.^{11,23} We were able to utilize tumor acidosis in a clinical setting, in diverse cancer types. Second, although the unique features of nanoscale cooperativity and non-covalent self-assembly have been long recognized, they have not been clinically exploited by a systemically administered agent. The salient features of nanoscale cooperativity, based on macromolecular non-covalent interactions, include: weak and polyvalent interactions driving self-assembly; the creation of larger structures that cannot be easily synthesized by covalent chemistry; faster responses to environmental stimuli due to the reduced energy barriers, as occurs with covalent bonds; and, most importantly, emergent properties where the system behavior cannot be predicted by studying the individual components in isolation.¹⁸

Our *ex vivo* spraying experiments give additional proof of the specificity of the imaging agent, as these results indicate that no other mechanism, such as the Enhanced Permeability and Retention (EPR) effect, other than pH is responsible for fluorescent activation. This was illustrated by the fact that tumor specific fluorescence was observed after topical administration of ONM-100, emphasizing that the EPR effect *in vivo* is responsible for the selective accumulation of micelles in the tumor, but the pH of the tumor is responsible for the opening of the micelle and the activation of the fluorescence signal. Moreover, mechanical destruction of the agent can be excluded since no fluorescence was observed in the plasma samples of patients without acidification.

A potential drawback might be the activation of ONM-100 by other mechanisms associated with a lowered pH, such as in inflammatory tissue.²⁴ Some tumors present with a peri-tumoral inflammatory rim as part of the defense mechanism of the innate immune system to the invading tumor cells.²⁵ In this study, we did not observe this phenomenon in this small series of specimens. However, we believe that, if this occurs, it has a minimal impact, and it might actually provide a fluorescent rim surrounding the actual tumor-positive margin, and thus serve the clinical applicability in fluorescence-guided surgery.

Biomarkers like HIF-1- α , CA-IX and VEGF-A, among others, all play a significant role in the development and maintenance of tumor acidosis.^{11,26,27} As activation of ONM-100 relies on tumor acidosis in general, and is not dependent on one specific biomarker, immunohistochemical staining would only give limited information on the molecular mechanism of fluorescence activation. It would be beneficial to correlate the *in vivo* data with the microscopic distribution of the fluorescence data. Unfortunately, this was not feasible in the current clinical trial since ONM-100 is not membrane-bound and the fluorescence was washed out during the standard of care processing of tissues. Invasive pH-measurements to correlate the localization of fluorescence with pH within and surrounding the tumor was deemed neither feasible nor safe in this Phase 1 clinical trial due to the following reason: undertaking pinpoint invasive pH measurements in the tumor can influence the integrity of the surgical specimen, potentially hampering standard of care histopathological evaluation. A possible remedy for this, in future studies, could be pH-measurements with, for example, chemical exchange saturation transfer magnetic resonance imaging (CEST-MRI) in patients who receive ONM-100.²⁸

The timing of the administration of ONM-100 was kept consistent, to 24 hours prior to surgery in this Phase 1 feasibility and imaging study, to prevent heterogeneity in the imaging data and was chosen based on pre-clinical data.¹⁷ A different imaging interval is being investigated in an ongoing Phase 2 study of ONM-100, since administration only a few hours prior to surgery might be preferable in terms of clinical implementation. In addition, higher dosages could be investigated in subsequent studies, since this might further improve TBRs. Moreover, our results indicate that ONM-100 could be utilized in a broad variety of other solid tumor types.

Clinical success can be improved when surgeons are provided with a more accurate and unambiguous delineation of the cancer location in addition to the extensive information on the locations of the tumors from conventional ultrasound, CT, PET or MR imaging. The ability of an optical imaging output to improve surgical outcomes is predicated on delivering information the surgeon does not have from pre-operative imaging and intraoperative inspection by vision and palpation alone.²⁹ The combination of tumor acidosis as a general phenomenon and the cooperativity of ONM-100 has the potential to adequately improve surgical guidance in a variety of tumors using fluorescence. The broader implication is that nanoscale macromolecular cooperativity can exploit a labile physiologic parameter for clinical purposes. Other biologic parameters (e.g., hypoxia, redox potential) which were previously unexploitable due to unpredictable variability may also be amenable to clinical targeting based on this chemical principle, representing a new therapeutic paradigm.

Nanoscale macromolecular cooperativity, which responds to pH changes from cancer acidosis, is demonstrated to be safe and clinical utility of the ONM-100 imaging agent for intraoperative and *ex vivo* detection of cancer is shown in this study. The results of our clinical study serve as a proof of principle that physiologic parameters such as pH, which were previously inaccessible therapy targets, may become clinically relevant through the application of macromolecular cooperativity.

METHODS

GMP synthesis of the pH-activatable micelles

Clinical grade pH-activatable ONM-100 was released from the Good Manufacturing Practice (GMP) facility of Bioserv Corporation, San Diego, USA under FDA regulated conditions. The product was shipped to PCI Pharma Services, Bridgend, UK for distribution to the Hospital Pharmacy Trial Bureau of the University Medical Center Groningen (UMCG), The Netherlands. A detailed description of the production process was published by a previous study. Briefly, ONM-100 consists of polymeric micelles labeled with IndoCyanine Green (ICG). Chemically, the ONM-100 drug substance comprises a diblock copolymer of polyethyleneglycol (PEG) (~113 repeating units) and a poly(methyl methacrylate) derivative covalently conjugated to functionalized ICG as the fluorophore. The ICG content was determined by a qualified method from its molecular weight of $37.5\text{kD} \pm 12.5\text{kD}$. Vials containing 9 mg ONM-100 drug substance formulated in sterile water for injection with 10% (v/w) trehalose were used to prepare infusions at a concentration of 3 mg/ml. After the final product was released by the certified qualified person (QP) at the UMCG Hospital Pharmacy facility, the imaging agent was intravenously administered to the subjects.

Subject population

The subjects included in this first-in-human clinical study had histologically proven primary or recurrent HNSCC, BC, EC or CRC. Subjects were eligible if they were 18 years of age or older, and their hematological status was acceptable as was their kidney and liver function. The subjects had to abstain from alcohol intake during the study period (from 24 hours before to 17 days after imaging agent administration). Any subjects receiving neoadjuvant therapy prior to surgery were excluded from Phase 1a. Other exclusion criteria were: an inability to give informed consent; participation in another clinical trial with an investigational product; inadequately controlled hypertension; history of allergic or infusion reaction to iodine, iodine-based contrast, shellfish or ICG; those receiving potentially highly hepatotoxic medication; pregnancy; and subjects with magnesium, potassium and calcium lower than the lower normal limit.

Clinical trial design

This dose-finding Phase 1 study was performed in two medical centers and enrolled 30 subjects from March 2018 until December 2018. The study, conducted in both the University Medical Center Groningen (UMCG, Groningen, The Netherlands) and the Martini Hospital Groningen (MZH, Groningen, The Netherlands) was approved by the Institutional Review Board (IRB) of the UMCG (METc Number 2017/580).

The study was conducted according to the Dutch Act on Medical Research involving Human Subjects (WMO) and to the principles of the Declaration of Helsinki (adapted version Fortaleza, Brazil, 2013). All the subjects were identified by the respective multi-disciplinary tumor boards of the participating hospitals. After a pre-screening, the eligible subjects were orally informed and received written information about the study. All the participants gave written informed consent before the start of any related study procedure. An independent medical monitor was assigned to review the screening safety assessments prior to enrollment. Serious adverse events, if present, were immediately reported to the IRB, the medical monitor and the Dutch central committee on research involving human subjects (CCMO) and were followed up until resolved or a stable medical situation was achieved. The trial was registered at the Netherlands National Trial Register (NTR number 7085) and within the European Clinical Trials Database (EudraCT 2017-003543-38).

Primary outcomes were safety, pharmacokinetics and imaging feasibility of ONM-100. Secondary outcomes were to determine a range of safe doses of ONM-100 for intra-operative imaging using commonly used fluorescence camera systems. The complete study was divided in two phases, Phase 1a and Phase 1b respectively. We adhered to the FDA guidelines (Guidance for Industry, Developing Medical Imaging Drug and Biological Products, Part 2 Clinical Indications) when designing the Phase 1a dose-finding protocol, followed by setting a dose expansion cohort (15 subjects, Phase 1b). Five different doses were used in an escalating/de-escalating scheme (i.e., 0.3 mg per kg, 0.5 mg per kg, 0.8 mg per kg, 0.1 mg per kg and 1.2 mg per kg) and administered to three subjects each. In Phase 1a, only subjects with histologically proven HNSCC and BC were included to minimize tumor heterogeneity and to collect information about the potential tumor agnostic characteristics of ONM-100. After dosing each cohort, a dose escalation meeting was held with the principal investigators, sub-investigators and the sponsor's medical monitor to make sure the escalation to a higher dose was safe. The most optimally performing dose was 1.2 mg per kg in Phase 1a, based on tumor visualization and safety data. The additional 15 subjects included in Phase 1b were studied to confirm the safety and to evaluate the tumor agnostic properties of ONM-100 imaging in additional solid tumor types (EC, CRC) to HNSCC and BC. In both phases, the subjects received a single dose of ONM-100 24±8 hours prior to surgery. All doses were injected intravenously at 15 mg per min. After the injection, the infusion line was flushed with 5ml sterile water.

Safety measurements

A primary outcome of our study was to measure the safety of ONM-100. The subjects underwent a medical screening procedure before enrollment in the study, consisting of vital signs measurements, a physical examination, a standard 12-lead electrocardiogram (ECG) and laboratory tests (including a serum pregnancy test of women of childbearing potential). Once enrolled, an ECG was performed 1h and 10 days after the ONM-100 infusion (the latter only in Phase 1a). Lab tests were performed before and after administration (day 1, 2, 3 and 10). The vital signs and physical examinations were measured before and after imaging agent injection (5min, 1h, day 2, 3 and 10). The subjects were asked about signs and symptoms before and after the

injection (5min, 1h, 3h, 8h, day 2, 3, 10 and 17). Standard postoperative checkups were arranged for within two weeks after surgery. During these visits, wound healing and adverse events were monitored as well. Adverse event assessment was performed according to the National Cancer Institute CTCAE version 4.0.

Pharmacokinetic assessments

An additional primary outcome was to measure the pharmacokinetics of ONM-100. Blood samples were collected for pharmacokinetic (PK) analysis prior to, and after 10min, 30min, 1h, 3h, 8h, 24h, 48h, 72h and 240h of intravenous administration of ONM-100. The blood was collected in 4 mL BD K2EDTA vacutainers and stored directly on ice. The samples were then centrifuged at 1500x g for 10 minutes and divided into three vials under cold conditions. The vials were stored in a -80 degrees Celsius freezer in the UMCG and then transported on dry ice to Intertek Pharmaceutical Services (San Diego, California, United States of America).

The ONM-100 plasma concentrations were determined using a validated direct fluorescence reader assay (Intertek Pharmaceutical Services, San Diego, California, United States of America) and the PK analysis was performed by Pacific BioDevelopment (Davis, California, United States of America). Plasma concentration versus time profiles were generated for each subject. The PK parameters were estimated using Phoenix WinNonlin (version 8.0). The estimated parameters were C_{10m}, C_{max}, T_{max}, AUC_{last}, AUC_{0-24hr} and AUC_{0-24hr}. Values below the level of quantitation (<10ug/ml, BQL) were set to 0.

The linear trapezoid method was used for the estimation of the area under the plasma concentration versus time curves from dosing to the last time point with a measurable concentration (AUC_{last}). The last three or more time points were used to estimate the elimination rate constant (λ_z) which was used to estimate the terminal-phase half-life (T_{1/2}).

Surgical procedure (standard of care)

All the subjects underwent surgical removal according to the standard surgery protocols of both hospitals for each respective tumor type. Dependent on the tumor type and/or stage, a sentinel lymph node biopsy (lymph node mapping using ^{99m}Tc and perioperative detection using a gamma-probe) or a lymph node dissection was performed on some of the subjects. Based on prior experience using fluorescence-guided surgery at the UMCG and MZH, there was minimal interference with the standard of care. As described earlier, the use of methylene blue was avoided and the use of fluorescent skin markers and (green) fluorescent sterile drapes was minimized due to the potential interference with ONM-100.²¹

Intraoperative fluorescence imaging devices

A secondary outcome of our study was to investigate different imaging systems. Two open-surgery intraoperative fluorescent camera systems were used in this study to detect ONM-100, namely the Explorer Air® (SurgVision B.V., Groningen, The Netherlands) and the SPY Elite® (Stryker, Kalamazoo, MI, USA). When performing minimally invasive surgery (e.g., robot assisted

esophagectomy or diagnostic laparoscopic surgery for peritoneal metastasis), clinically available near-infrared (NIR) imaging systems were used namely, the Olympus NIR Laparoscope (Olympus, Sjinjuku, Tokyo, Japan) and the Intuitive Da Vinci Firefly robot NIR laparoscope (Intuitive Surgical, Sunnyvale, CA, USA).

The Explorer Air®, which provides real-time simultaneous fluorescence and white light (color) images, is currently undergoing CE-marking in Europe and is only available in the European and US markets for experimental use. Fluorescence is excited by NIR light emitting diodes (LEDs) with an excitation peak of 760nm. Filtered white light is used to illuminate the color images. A software user interface enables the user to control the camera settings and to display the color and fluorescence images. The output is that both still images and movies can be recorded and stored in a TIFF format. Although the excitation peak is not optimized for ICG detection, ICG is efficiently detected due to overlapping light spectra. The working distance of the imaging system was set at 20cm above the surgical field. All images were obtained with a fluorescence exposure time of 100ms and a 100 gain. If over-saturation occurred, the exposure time was lowered to 50ms or 25ms but if it persisted, the gain was lowered to 10.

The SPY Elite provides real-time fluorescence and white light imaging. The system is CE-marked for ICG detection and is commercially available. NIR light from the illumination module in the imaging console is transmitted to the imaging head via fiber-optic cables. The SPY Elite has an excitation peak of 805nm. A software user interface enables the user to control the camera settings and to capture white light images and fluorescence movies. The working distance of the imaging system was set at 30cm above the surgical field. All images were obtained with a frame-rate of 7.5 per second. White light images and fluorescence movies were recorded and stored from the SPY Elite in AVI/PNG format.

The Da Vinci Firefly and the Olympus NIR Laparoscope provide real time fluorescence and white light images. Both systems, which have an excitation peak of 805nm, are CE-marked for ICG detection and are commercially available. A software user interface is provided. The working distance of the imaging system was set at 2-20cm above the surgical field. As described earlier, all the intraoperative camera systems were calibrated using a calibration disk (CalibrationDisk®, SurgVision BV, The Netherlands). The ICG used in the CalibrationDisk® was stabilized by dissolving it in methanol and it was then diluted to different concentrations ranging from 0.005 mg per ml to 1.5 mg per ml to check whether the systems could detect low and high fluorescent signals and as a diagnostic test of the systems.

Fluorescence imaging systems for *ex vivo* imaging

To further validate the intra-operative imaging feasibility as an primary outcome, we used the the closed-field macroscopic fluorescence PEARL-trilogy® imaging device (Li-COR BioSciences Inc., Lincoln, NE, USA), which is designed for *ex vivo* fluorescence imaging. A charge-coupled device (CCD) camera detects fluorescence in the NIR wavelength with a peak emission at 785 nm. The 11.2 cm x 8.4 cm field of view and the focus point can be adjusted based on specimen height. The same resolution setting was used (85 µm) for all the specimens throughout the study.

Intraoperative imaging procedures

A secondary outcome of our study was to investigate different imaging systems. All standard of care surgical procedures had priority over study related procedures. Fluorescence imaging was performed at pre-defined time points: i) just before the first incision; ii) when tumor visibility was most optimal, as judged by the surgeon (e.g., after lump preparation for a lumpectomy); iii) after excision of the the surgical cavity to inspect for remaining fluorescent spots. If fluorescent spots were detected, the surgeon was allowed to biopsy these spots, and iv) on all the resection planes after excising the specimen to check for the presence of fluorescent spots. An intraoperative fluorescence margin assessment entails a combination of the fluorescence image of the surgical cavity and the fluorescence image of the excised specimen within 1 hour after excision. The surgeon was able to look at a second monitor to evaluate the fluorescence images while performing the surgical procedure. During the imaging procedure, the ambient light in the surgical theatre was switched off to prevent possible interaction with the fluorescence imaging procedure itself. The standard of care was not influenced or altered by the imaging procedures. Regarding intrathoracal esophageal imaging, only extraluminal fluorescence imaging of the tumor and resection plane was performed.

In vivo TBR was calculated for all the HNSCC subjects whose mucosal tumors had been directly exposed to the intraoperative camera. We only calculated the *in vivo* TBRs for the HNSCC subjects because the other tumors (BC, EC, CRC) were deeper-seated and thus a) not visible or b) no reliable calculation could be done due to overlying tissue and differences in optical tissue properties. The Mean Fluorescence Intensity (MFI) of both the tumor and background areas was calculated. The MFI was based on a Region of Interest (ROI) which was carefully determined based on a macroscopic examination. The TBR was calculated as Tumor ROI (MFI tumor) / Background ROI (MFI non-tumor tissue) per tissue slice. The median TBR was calculated on a per subject base. The data (MFI, TBR) were plotted as graphs using GraphPad Prism, version 8.

Specimen handling

According to the SOC, the specimen was marked using sutures to aid orientation during tissue processing and cross-correlation with the histopathology immediately after excision. The exact location of the suture was tumor type and surgeon dependent and was carefully documented to correlate fluorescence signals with tissue orientation.

(Freshly) excised surgical specimen imaging procedures

The specimens were stored, as much as possible, in the dark during all the tissue processing phases to prevent possible photobleaching of the imaging agent. All the surgical specimens were handled to conform to the standard of care which was not affected by study related procedures. Immediately after excision all six resection planes of the specimen were imaged, size allowing, (e.g., frontal, dorsal, lateral, medial, caudal and cranial) by both the intraoperative camera system of choice as well as the PEARL-trilogy® system with a maximum duration of 60 minutes after surgical excision of the specimen. The combined imaging time of both devices was a maximum

of 5 minutes. The specimens' resection planes were marked with blue and black ink. The use of other ink colors was avoided since these might interfere with the fluorescence in the NIR range. The restricted use of two colors of ink did not affect the standard of care for tissue processing by the pathologist but, if a third ink color was needed, green ink was used to define additional pathological resection margins of interest.

The fluorescence imaging time points were adapted due to the SOC differences in specimen processing of the different tumor types. Briefly, the fresh BC specimens were sliced on the day of surgery and then formalin fixed whereas the other tumor types were sliced after formalin fixation of the whole resection specimen 1-3 days after surgery. The surgical specimen was serially sliced into ± 0.5 cm thick tissue slices. White light photographs were made during and directly after slicing for orientation purposes. After slicing, both sides of each tissue slice underwent fluorescence imaging in a light-tight environment (PEARL-trilogy®). The BC slices were therefore imaged approximately 120min after excision. The other tumor types were imaged after formalin fixation and thus 1-3 days after excision.

Subsequently, a pathologist blinded for the recorded fluorescence images, examined the tissue slices macroscopically. This involved a gross examination by visual inspection and palpation alone and the selected regions of interest were embedded in paraffin blocks for further standard of care histological analysis. Other tissue samples that had not been selected by gross examination were embedded based on high fluorescent intensity spots or regions. In line with Koller et al., a standardized workflow was executed in order to cross-correlate the final histopathology results with the recorded fluorescence images of the tissue slices of interest. Subsequently, Hematoxylin / Eosin (H/E) stained 4 μ m sections were produced and analyzed by a board-certified pathologist who was also blinded for the obtained fluorescent imaging data.

Quantification of ONM-100 fluorescence in tissue slices

To further validate the intra-operative imaging feasibility as an primary outcome , the images of both sides of the respective tissue slices collected with PEARL-trilogy® were used to calculate the Mean Fluorescence Intensity (MFI) of both tumor and background areas. The MFI was based on a Region of Interest (ROI) which was carefully determined on the H/E 4 μ m slides by a board-certified pathologist blinded for the fluorescent imaging data and subsequently overlaid precisely on the corresponding tumor and normal tissue slices. Non-tumor tissue was considered to be any other tissue than the tumor tissue in the respective tissue slice. No distinction was made between specific non-tumor tissue types when defining the background. TBR was calculated as Tumor ROI (MFI tumor) / Background ROI (MFI non-tumor tissue) per tissue slice. A median TBR was calculated on a per subject base. The data (MFI, TBR) were plotted as graphs using GraphPad Prism, version 8.

Specific *ex vivo* activation experiments

An activation buffer (0.1M sodium acetate-acetic acid) or phosphate-buffered saline (PBS) was added to human plasma with a certain concentration of ONM-100 and mixed with 20X PBS in a 96-well plate. The fluorescence was measured using a plate reader (TECAN Infinite M200 PRO, Männedorf, Switzerland).

The tumor was collected with adjacent stromal tissue during surgery and immediately frozen in optimal cutting temperature compound (OCT). It was cut into 8µm frozen sections and sprayed with a fluorescent pH sensor (ICG was substituted with a tetramethylrhodamine (TMR) dye) and incubated for 3 minutes. After 10 min of fixation with formalin, the slides were washed three times with 0.9% NaCl + 0.5% Tween 20. The sections were DAPI stained and scanned for fluorescence (Zeiss Axio slide scanner, Oberkochen, Germany). Adjacent slides were H/E stained for histopathological correlation purposes.

Statistical analysis

The H/E sections, as shown in the respective figures, correspond to the tissue as shown in the adjacent panels. Note that from all included tissue slides, a corresponding H/E section has been cut and evaluated by the pathologist for correlating the fluorescence images to histopathology. The MFI was calculated, using Image J Fiji, as total counts per ROI pixel area in both the tumor and background non-tumor tissues. The data was tested for Gaussian distribution using Anderson-Darling and Shapiro-Wilk tests; none of the data were normally distributed. Differences in fluorescence intensities between dose cohorts were tested using a Wilcoxon statistical test. The data are presented as median and interquartile ranges. A Receiver-Operator-Curve (ROC) was calculated using Graphpad Prism, version 8. A P value <0.05 was regarded as statistically significant. GraphPad Prism, version 8 was used for the statistical analyses.

Dose dependent increase of exposure of ONM-100 **Supplementary Figure 1 | Dose dependent pharmacokinetics**

There is a dose-dependent correlation between the mean plasma concentration of ONM-100 ten minutes after administration and the mean area under the curve in the first 24 hours with a R^2 of 0.95 and 0.98 respectively. Dots represent mean values per cohort, error bars standard deviation. $N=3$ for 0.3, 0.5 and 0.8 mg per kg cohorts, $N=18$ for 1.2 mg per kg cohort. Abbreviations: C10m: Plasma concentration at 10 minutes; AUC0-24h: Area under the curve in the first 24 hours; ug: micrograms; mL: milliliters; hr: hour. Source data are provided as a Source Data file.

Supplementary Figure 2 | Pharmacokinetic comparisons of different tumor types

There were no apparent pharmacokinetic differences between the subjects who received 1.2 mg per kg, based on tumor type. The mean terminal-phase half-life in the 1.2 mg per kg cohort was 44.5 with a standard deviation of 15.8 hours. Dots represent mean values per tumor type (N=3-13, depending on number of patients), error bars standard deviation. Abbreviations: BC: Breast Cancer; HNSCC: Head and Neck Squamous Cell Cancer; EC: Esophageal Cancer; CRC: Colorectal Cancer. Source data are provided as a Source Data file

Supplementary Figure 3 | Fluorescence microscopy to confirm tumor-specific activation of ONM-100

Fluorescence microscopy was performed ex vivo after spraying ONM-100 onto tissue sections of a freshly frozen HNSCC specimen directly after excision. DAPI was applied for nuclear staining (blue) (a) and ONM-100 (green) for fluorescence visualization (b). A sharp delineation of fluorescence between the tumor and stromal tissue (c) was observed and correlated with corresponding histopathology tissue sections stained with hematoxyline and eosin (d). Abbreviations: HNSCC: Head and Neck Squamous Cell Carcinoma; T: Tumor; S: Stroma; H/E: Hematoxylin Eosin; DAPI: 4',6'-diamidino-2-phenylindole. Experiment has been repeated three times.

Supplementary Figure 4 | pH-dependent activation of ONM-100 in human plasma

Increasing amounts of ONM-100 were added to human plasma which did not show an increase in fluorescence. When the experiment was repeated after the addition of HCl to supply protons to the plasma, there was an increase in fluorescence with the addition of increasing amounts of intact ONM-100 suggesting that acidosis was activating the ONM-100 and thus the fluorescence in a dose-dependent manner. Dots represent individual data points. Error bars represent standard deviation. Abbreviations: RFU: Relative Fluorescence Units.

Supplementary Figure 5 | Clinically relevant images

Intraoperatively detected Peritoneal Metastasis (PM) (a-c). An additional tumor lesion detected in the surgical cavity after a Head and Neck Squamous Cell Carcinoma (HNSCC) resection of the mandible (d-f). A false positive fluorescent lesion from salivary gland tissue (g-i). Additional satellite metastases of the primary tumor lesion were detected in two BC subjects and confirmed by final histopathological examination (j-o). An additional primary tumor lesion was detected on a fresh tissue slice from a BC subject showing triple negative breast cancer which was not detected before and during surgery (p-r). The tumor is delineated as a solid black line in the H/E slices (c,f,l,o,r). The false positive contained no viable tumor tissue (i). Abbreviations: H/E: Hematoxylin Eosin; BC: Breast Cancer.

Supplementary Figure 6 | Representative images per subject (previous two pages)

Representative sequences of white light and fluorescent images of tissue slices with the corresponding H/E 4um slice per subject. A: 0.1 mg per kg dose cohort. HNSCC, subject 1 (a-c). HNSCC, subject 2 (d-f). HNSCC, subject 3 (g-i); the solid line on the H/E slice represents the tumor. B: 0.3 mg per kg dose cohort. BC, subject 4. The viable tumor is between the solid and dashed line on the H/E slice. The dashed line represents the necrotic area on a H/E slice (a-c). BC, subject 5 (d-f). BC, subject 6. The arrow indicates a tumor-positive surgical margin (g-i). The solid line on the H/E slice represents the tumor. C: 0.5 mg per kg dose cohort. HNSCC, subject 7 (a-c). BC, subject 8 (d-f). HNSCC, subject 9 (g-i). The solid line on the H/E slice represents the tumor. D: 0.8 mg per kg dose cohort. BC, subject 10 (a-c). HNSCC, subject 11 (d-f). BC, subject 12 (g-i). The solid line on the H/E slice represents the tumor. E: The MFI on all the subjects' tumor tissue slices was higher compared to non-tumor tissue. Dots and asterisks represent median values

($N \geq 3$ -8, depending on availability of tissue slides), error bars represent interquartile ranges. F: 1.2 mg per kg dose cohort. HNSCC, subject 13 (a-c). HNSCC, subject 14 (d-f). BC, subject 15 (g-i). The solid line on the H/E slice represents a tumor. G: 1.2 mg per kg dose cohort. BC, subject 16 (a-c). HNSCC, subject 17 (d-f). HNSCC, subject 18 (g-i). The solid line on the H/E slice represents a tumor. The dashed line on the H/E slice represents a Ductal Carcinoma in Situ. H: 1.2 mg per kg dose cohort. CRC subject 19 (a-c). HNSCC, subject 20 (d-f). No vital tumor present after neo-adjuvant treatment of EC therefore complete response, subject 21 (g-i). The solid line on the H/E slice represents a tumor. I: 1.2 mg per kg dose cohort. BC, subject 22 (a-c). EC, subject 23 (d-f). HNSCC, subject 24 (g-i). The solid line on the H/E slice represents a tumor. J: 1.2 mg per kg dose cohort. EC, subject 25 (a-c). HNSCC, subject 26 (d-f). BC, subject 27 (g-i). The solid line on the H/E slice represents a tumor. K: 1.2 mg per kg dose cohort. CRC subject 28 (a-c). CRC, subject 29 (d-f). BC, subject 30 (g-i). The solid line on the H/E slice represents a tumor. Abbreviations: H/E: Hematoxylin Eosin, HNSCC: Head and Neck Squamous Cell Carcinoma, BC: Breast Cancer, EC: Esophageal Cancer, CRC: Colorectal Cancer. TBR: Tumor to Background Ratio. MFI: Mean Fluorescence Intensity, IQR: Interquartile range.

Supplementary Figure 7 | Correlating fluorescence surgical margin assessment with final histopathology results

Intraoperative assessment of the surgical margin during fluorescence-guided surgery can be done either by intraoperative fluorescence imaging of the surgical cavity or fluorescence imaging of the excised specimen at the 'Back-Table'. The final thology is correlated with the fluorescence images of breast cancer subjects (a) and head and neck squamous cell carcinoma subjects (b). Abbreviations: BC: Breast Cancer, HNSCC: Head and Neck Squamous Cell Carcinoma.

Supplementary Figure 8 | In vivo imaging using ONM-100 fluorescence

Representative examples of in vivo imaging data using ONM-100 fluorescence. A large tongue carcinoma with a central necrotic ulcer was in vivo visualized using ONM-100 (a). A cancer located at the right mandible / floor of mouth was in vivo visualized using ONM-100 (b). A large tongue carcinoma with a central necrotic ulcer was in vivo visualized using ONM-100 (c). A colorectal carcinoma with extensive peritoneal metastases was in vivo visualized using ONM-100 (d).

Supplementary Table 1 | Patient characteristics

Patient characteristics. Abbreviations: TNM; Tumor Nodal Metastasis tumor classification. BC: Breast cancer, HNSCC: Head and Neck squamous cell carcinoma; PC: Peritoneal metastasis; EC: Esophageal cancer; OC: Ovarian Cancer; RC: Rectal Cancer.

Patient ID	Dose (mg per kg)	Weight (kg)	Absolute tracer dose (mg)	Tumor Type	Stage (TNM)	Max. tumor size
ON1101	0.3	83.5	25.05	BC	pT2N3aM1	3.0cm
ON1102	0.3	113	33.9	BC	pT1cNo	1.1cm
ON1103	0.3	71	21.3	BC	pT2No	2.5cm
ON1104	0.5	92	46	HNSCC	pT4N1mo	3.0cm
ON1105	0.5	52	26	BC	pT2No	2.8cm
ON1106	0.5	119.2	59.6	HNSCC	pT3N2bM0	5.0cm
ON1107	0.8	93.7	74.96	BC	PT3N3a	6.5cm
ON1108	0.8	93	74.4	HNSCC	pT3NoMx	3.2cm
ON1109	0.8	73	58.4	BC	pT1cNo	1.1cm
ON1110	0.1	64	6.4	HNSCC	pT3Nomx	2.3cm
ON1111	0.1	53	5.3	HNSCC	pT1NoMx	2.0cm
ON1112	0.1	69	6.9	HNSCC	pT2No	1.4cm
ON1113	1.2	65	78	HNSCC	pT3N2bMx	4.0cm
ON1114	1.2	64	76.8	HNSCC	PT1NiMx	2mm
ON1115	1.2	64	76.8	BC	pT1bNoMx (2 primary tumors)	0.6cm
ON1116	1.2	72	86.4	BC	pT1cNo	1.1cm
ON1118	1.2	72	86.4	HNSCC	pT2No	2.4cm
ON1119	1.2	89	106.8	HNSCC	pT4No	3.8cm
ON1120	1.2	94	112.8	PC	n/a	n/a
ON1121	1.2	79	94.8	HNSCC	pT1No	5mm
ON1122	1.2	78	93.6	EC	ypT0No	n/a
ON1123	1.2	60	72	BC	pT1cNo	1.2cm
ON1124	1.2	96	115.2	EC	ypT3N1	5cm
ON1125	1.2	85	102	HNSCC	pT1No	1.7cm
ON1126	1.2	96	115.2	EC	ypT2N2	5cm
ON1127	1.2	46.5	55.8	HNSCC	pT2No	1.5cm
ON1128	1.2	87	104.4	BC + OC	pT3NoMo + OC: no tumor	1.5 cm CRC: 1.5cm RC: 12cm
ON1129	1.2	110	132	CRC + RC	ypT2No + pT3aNo	12cm
ON1130	1.2	83	99.6	CRC	pT4NiM1	Not applicable
ON1151	1.2	81	97.2	BC	pT1b	1cm

Supplementary Table 2 | Correlating fluorescence with histopathology per patient

Intraoperative assessment of the surgical margin during fluorescence-guided surgery, can be either done by intraoperative fluorescence imaging of the surgical cavity or fluorescence imaging of the excised specimen at the 'Back-Table' which is then correlated with the histopathology results on a per patient basis. The surgical resection margins are displayed according to the US Guidelines per tumor type. Regarding patient 11, no fluorescence imaging of the surgical cavity was performed due to logistics. Regarding patient 18, no biopsy was performed due to the severity of the remaining fluorescence. Regarding patient 30, no biopsy was performed due to logistics. Regarding patients 19, 29 and 21, no tumor containing specimens were excised since peritoneal metastases or a complete response after neoadjuvant treatment was observed respectively. Abbreviations: BC: Breast cancer; HNSCC: Head and neck squamous cell cancer; EC: Esophageal cancer; CRC: Colorectal cancer; DCIS: Ductal Carcinoma in Situ; TN: True negative; TP: True positive; FP: False positive.

Patient	Tumor type	Margin status	Cavity fluorescence	Biopsy performed	Specimen margin fluorescence	Correlation
1	BC	Tumor-negative	no		no	TN
2	BC	Tumor-negative	no		no	TN
3	BC	Tumor-positive	no		yes	TP
4	HNSCC	Tumor-negative	no		no	TN
5	BC	Tumor-negative	no		no	TN
6	HNSCC	Tumor-positive	yes	yes	yes	TP
7	BC	Tumor-negative	no		no	TN
8	HNSCC	Tumor-negative	yes	yes	yes	FP
9	BC	Tumor-positive	yes		yes	TP
10	HNSCC	Tumor-positive	no		yes	TP
11	HNSCC	Tumor-positive	n/a		yes	TP
12	HNSCC	Tumor-negative	no		no	TN
13	HNSCC	Tumor-positive	yes	yes	yes	TP
14	HNSCC	Tumor-negative	no		yes	FP
15	BC	Tumor-negative	no		no	TN
16	BC	Tumor-negative	no		no	TN
17	HNSCC	Tumor-positive	no		yes	TP
18	HNSCC	Tumor-positive	yes	n/a	yes	TP
19	CRC	n/a	n/a		n/a	n/a
20	HNSCC	Tumor-negative	no		yes	FP
21	EC	n/a	n/a		n/a	n/a
22	BC	Tumor-negative	yes	yes	yes	FP
23	EC	Tumor-negative	no		no	TN
24	HNSCC	Tumor-negative	no		no	TN
25	EC	Tumor-negative	no		no	TN
26	HNSCC	Tumor-negative	no		no	TN
27	BC	Tumor-positive	no		yes	TP
28	CRC	Tumor-negative	no		no	TN
29	CRC	n/a	n/a		n/a	n/a
30	BC	Tumor-negative (DCIS)	yes	n/a	yes	FP?

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CHAPTER 4

A standardized framework for fluorescence-guided margin assessment for head and neck cancer using a tumor acidosis sensitive optical imaging agent

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ABSTRACT

Intra-operative management of the surgical margin in patients diagnosed with head and neck squamous cell carcinoma (HNSCC) remains challenging as surgeons still have to rely on visual and tactile information. Fluorescence-guided surgery using tumor-specific imaging agents can assist in clinical decision-making. However, a standardized imaging methodology is lacking. In this study, we determined whether a standardized, specimen- driven, fluorescence imaging framework using ONM-100 could assist in clinical decision-making during surgery.

Thirteen patients with histologically proven HNSCC were included in this clinical study and received ONM-100 24 ± 8 hours before surgery. Fluorescence images of the excised surgical specimen and of the surgical cavity were analyzed. A fluorescent lesion with a tumor-to-background ratio (TBR) > 1.5 was considered fluorescence-positive and correlated to standard of care (SOC) histopathology.

All six tumor-positive surgical margins were detected immediately after excision using fluorescence-guided intra-operative imaging. Postoperative analysis showed a median TBR ($\pm$ IQR) of the fluorescent lesions on the resection margin of 3.36 ± 1.62 . Three fluorescence-positive lesions in the surgical cavity were biopsied, and showed occult carcinoma and severe dysplasia, and a false positive fluorescence lesion.

Our specimen-driven fluorescence framework using a novel, pH-activatable, fluorescent imaging agent could assist in reliable and real-time adequate clinical decision-making showing that a fluorescent lesion on the surgical specimen with a TBR of 1.5 is correlated to a tumor-positive resection margin. The binary mechanism of ONM- 100 allows for a sharp tumor delineation in all patients, giving the surgeon a clinical tool for real-time margin assessment, with a high sensitivity.

INTRODUCTION

Optimal surgical management of head and neck squamous cell carcinoma (HNSCC) requires a tumor resection with adequate surgical margins as inadequate surgical margins are associated with an increased chance of local recurrence and metastasis, eventually causing reduced overall survival.¹⁻³ Fluorescence-guided surgery (FGS) is a novel imaging technique that allows tumor visualization during surgery by targeting tumor-specific biomarkers, proteins or receptors. Multiple clinical phase 1 and phase 2 studies have investigated a variety of fluorescent imaging agents to enhance tumor detection, showing the potential of tumor-targeted FGS to guide surgical decision making in a variety of clinical settings.^{4,5} However, the clinical translation of fluorescence-guided margin assessment lacks a standardized imaging method that allows immediate and reliable feedback for the surgeon. In this study, we provide an *ex vivo* specimen driven imaging methodology in patients with HNSCC by implementing a promising pH dependent, tumor type-agnostic imaging agent called ONM-100.⁶ We suggest that *ex vivo* imaging might be preferable over *in vivo* imaging since external factors influencing imaging results are limited. Subsequently, we hypothesize the potential clinical effects of this standardized method for our patient cohort.

In recent years, a variety of tumor-specific fluorescence optical imaging agents have been developed. Different targeting strategies, like fluorophores conjugated to monoclonal antibodies (MoAb) or PARP1-inhibitors, have been clinically evaluated in HNSCC.^{5,7-9} Despite interesting results, target heterogeneity within cancer and target expression on the majority of healthy cells might cause false positive signal and a limited discriminative strength. Moreover, as the half-life of a MoAb is on average 7-10 days, administration needs to be done 2-5 days prior to surgery, which comes with logistical challenges.^{5,10} The optical imaging agent ONM-100, consisting of micelles containing Indocyanine Green (ICG), can overcome these limitations by targeting more ubiquitous tumor characteristics, namely tumor acidosis.¹¹ The mechanism of action of the imaging agent is described previously.¹¹ Briefly, the micelles dissociate in the acidic tumor microenvironment, allowing the ICG to unquench and become fluorescent. As non-tumor tissue has a physiological pH, no 'background' fluorescence activation is observed in non-tumor tissue, resulting in high tumor-to-background ratios (TBR). The working mechanism has recently been clinically evaluated in 30 patients, showcasing the applicability of ONM-100 in four different tumor types.⁶

As the field of FGS is rapidly expanding, data collection and implementation of fluorescence-guided surgery into the clinical workflow needs to be standardized, since results are highly affected by factors other than the distribution of the fluorophore itself. Therefore, we validate a novel framework using a pH-activatable optical imaging agent in HNSCC (Supplemental Figure S1). We hypothesize that our standardized framework with a specimen-driven approach, combined with the next generation pH-activatable optical imaging agent, allows for reliable *real-time* intra-operative decision making. In this study, we present data of 13 HNSCC patients who received ONM-100 24 hours prior to surgery. Our data support the potential clinical value of this standardized framework using ONM-100 to alter *real-time* clinical decision making, since all tumor-positive surgical margins were detected immediately after excision in the *ex vivo* environment.

MATERIALS AND METHODS

Study Design

This study was conducted at the University Medical Center Groningen (UMCG) and data was analyzed retrospectively. The clinical study was approved by the Institutional Review Board (IRB number 2017/580) of the UMCG. Patients with histopathologically proven head and neck squamous cell carcinoma (HNSCC) scheduled for surgical removal were enrolled as part of a larger clinical trial (the Netherlands National Trial Register 7085, EudraCT number 2017-003543-38) assessing the safety and generic applicability and optimal dose of ONM-100. All patients gave their informed consent after being informed about the study. In the current study, we investigated if ONM-100 was suitable for intra- operative margin assessment in HNSCC surgery using a standardized fluorescence workflow (Supplemental Figure S2).

ONM-100 conjugation

ONM-100 consists of polymeric micelles labeled with IndoCyanine Green (ICG), as described previously.¹¹ ONM-100 was administered intravenously 24h $\pm$ 8 hours before surgery in different dosages, which has been extensively described in our previous study.⁶

Standardized method for fluorescence-guided clinical decision making

Specimen driven margin assessment

The excised surgical specimen of all patients was retrospectively analyzed using the PEARL-trilogy® imaging device (Li-COR BioSciences Inc., Lincoln, NE, USA). This closed-field device is suitable for *ex vivo* tissue imaging, as it eliminates ambient light and enables standardization of imaging between specimens. The tumor and all resection planes of the specimen were imaged immediately (<10 minutes) after tumor excision. The tissue thickness varied in size up to 10 centimeters. Imaging resolution was 85µm. If a fluorescent lesion was detected at one of the resection planes, this lesion was manually delineated and considered fluorescence-positive if a TBR of > 1.5 was obtained. This was based on an earlier reported study which determined 1.5 as adequate for discrimination between tumor and non-tumor tissue in FGS.¹² The fluorescent lesion was macroscopically delineated and the remaining, non-fluorescent, area of the respective resection plane was considered as background to determine the Mean Fluorescence Intensity (MFI). For each patient, MFI lesion / MFI background was used to determine the TBR on the resection planes. If a fluorescent lesion was detected, the location of the fluorescent lesion was correlated based on anatomical information with fluorescence images of the surgical cavity. For orientation of the surgical specimen, sutures were applied on the specimen to allow for maximal orientation, as per standard of care. For one patient, the specimen was too large for the sample stage of the PEARL-trilogy® imaging device. For this patient, the SurgVision Explorer Air® Vault was used (details described below).

Surgical cavity driven margin assessment

For the detection of remaining tumor in the surgical cavity, fluorescence imaging using two intra-operative camera devices, the SurgVision Explorer Air® (SurgVision B.V., Groningen, The Netherlands) and the NovaDaq Spy Elite (Stryker, Kalamazoo, MI, USA) was performed *in vivo*. All

additional fluorescent lesions detected prior to surgery or after surgery in the surgical cavity were biopsied, based on the definition of TBR > 1.5 and analyzed for final histopathological examination if deemed safe and feasible by the attending surgeon. *in vivo* imaging took approximately 5-10 minutes per procedure.

Ex vivo correlation of intra-operative fluorescence results

To validate fluorescence imaging results, fluorescence results were correlated with final histopathology. Following SOC, the surgical specimen was formalin-fixed for at least 24 hours and subsequently sliced in approximately 0.5cm thick tissue slices. In the case a fluorescent lesion was detected on the surgical margin during imaging of the excised specimen, this was precisely correlated to the representative tissue slice. Next, tissue slices were embedded into Formalin Fixed Paraffin Embedded (FFPE) blocks according to SOC by a pathologist blinded for fluorescence results. Fluorescent areas at the tissue slices that were not initially selected for histopathological analysis by the blinded pathologist, were additionally embedded. Histopathological assessment on Hematoxylin and Eosin (H&E) stained 3µm tissue sections was done by a board-certified pathologist, blinded for fluorescence and according to SOC.

Fluorescence imaging devices

In vivo fluorescence images were obtained using two intra-operative cameras, namely the Explorer Air® (SurgVision B.V., Groningen, The Netherlands) or the Novadaq SPY Elite® (Stryker, Kalamazoo, MI, USA). Fluorescence imaging with the SurgVision Explorer Air® was performed as previously described by our group. The Novadaq Spy Elite contains a Light Emitting Diode (LED) with a excitation wavelength of 805 nm, designed for the detection of ICG. White light images and fluorescence videos were obtained during surgery. The working distance above the surgical field was 30cm. Fluorescence gain could be manually adapted during imaging.

The excised whole specimen and tissue slices were imaged using the PEARL-trilogy® imaging device, using a CCD camera in the NIR wavelength (peak excitation 785nm, peak emission 820nm). The field of view of 11.2cm x 8.4cm and the focus point can be adjusted based on specimen height. All images were stored in TIFF format and post-processed by applying a color scheme for the benefit of fluorescence signal intensity visualization and analyzed using Fiji (version 2.0.0-rc-68/1.52h). The fluorescence values did not change by applying the color scheme.

RESULTS

Thirteen patients with histopathologically proven HNSCC were included. Patient and fluorescence imaging characteristics are depicted in Table 1.

Table 1 | Patient characteristics

Whole specimen fluorescence: Presence of a fluorescent lesion at the edge of the resected specimen. Surgical cavity fluorescence: Presence of a fluorescent lesion in the surgical cavity during surgical excision. + = positive for fluorescence, - = negative for fluorescence

Patient	Age	Location	Stage	Margin status	Closest margin	Specimen	Cavity
Patient 01	50	Mandible	pT4N1Mo	Close	3.5mm	-	-
Patient 02	69	Floor of mouth	pT3N2bMo	Positive	Cut through	+	-
Patient 03	53	Tongue	pT3NoMx	Close	3mm	+	+
Patient 04	46	Floor of mouth	pT3NoMx	Positive	<1mm	+	-
Patient 05	80	Cheek	pT1NoMx	Positive	Cut through	+	-
Patient 06	79	Cheek	pT2NoMx	Close	3mm	-	-
Patient 07	70	Tongue	pT3N2bMx	Positive	<1mm	+	+
Patient 08	48	Floor of mouth	pT1N1Mx	Free	>5mm	+	-
Patient 09	69	Mandible	pT2NoMx	Positive	<1mm	+	+
Patient 10	85	Mandible	pT4NoMx	Positive	Cut through	+	+
Patient 11	84	Palate	pT1NoMx	Close	3mm	+	-
Patient 12	58	Tongue	pT1NoMx	Close	2mm	+	-
Patient 13	77	Tongue	pT2NoMx	Free	>5mm	-	-

Ex vivo specimen driven margin assessment

Both the mucosal and the deep resection planes were evaluated by fluorescence imaging of the excised specimen (N=13, Figure 1 and Figure 2). All six histopathologically proven tumor-positive surgical margins (viable tumor cells ≤ 1 mm of margin) were detected using our standardized ex vivo fluorescence imaging framework, which enables accessible and fast identification of sharply delineated fluorescent signals (6/6, 100% sensitivity). Five out of these six tumor-positive surgical margins were located at the deep resection margin (Supplemental Figure S2). All fluorescent lesions correlated to the location of the histopathologically tumor-positive surgical margin with a median TBR of 3.36 ± 1.62 (Supplemental Figure S3). Additionally, five histopathologically proven close margins (viable tumor cells 1-5 mm of margin) were diagnosed in this study, of which three were determined as fluorescence-positive. Two were scored as fluorescence-negative, which had both a surgical margin 3 2mm (Table 1). Important to state, all surgical margins that were assessed as fluorescence-negative had a surgical margin > 2 mm. Of the two specimens that were

histopathologically diagnosed with tumor-negative surgical margins ($> 5\text{mm}$), one specimen was scored as fluorescence-positive at one of the resection margins, which was found to be caused by localized fluorescence around the salivary glands. This particular case showed no elevated fluorescence signal in the surgical cavity (Table 1). Fluorescence of the salivary glands was only observed in this single patient.

Figure 1 | Real-time surgical specimen analysis

Fluorescence-guided analysis of the mucosal tumor and of the deep resection margins in HNSCC patients was performed using Li-COR PEARL Trilogy. (a) Representative image of a mucosal tongue tumor with an insufficient superficial surgical margin (2mm) (b) Representative image of a mucosal tongue tumor with a sufficient superficial surgical margin ($>5\text{mm}$). (c) Representative image of a deep surgical resection margin negative for fluorescence which correlated with a tumor-negative margin (d) Representative image of a positive fluorescent lesion on a deep resection margin correlating with a tumor-positive surgical margin

Figure 2 | Tumor-positive surgical margins

Representative images of an excised tumor specimen, visualized on the mucosal site (a-b) which shows clear delineation of the tumor by fluorescence imaging. Next, the deep resection margin (i.e. basal surface) is shown (c-d), illustrating a sharply delineated fluorescence-positive lesion which correlated with a tumor-positive surgical margin of the respective specimen.

Surgical cavity driven margin assessment

During fluorescence imaging of the surgical cavity and peripheral surfaces in HNSCC (N=13), four fluorescent lesions could be identified of which in three cases a fluorescence-guided biopsy was performed. Three lesions were considered to be true positive. The mean TBR of these true positive biopsies was 4.68 (range 2.2 – 6.2) (Figure 3). *Patient 7* underwent tumor excision of a HNSCC located at the tongue/floor of the mouth. After initial tumor excision, a fluorescent lesion was detected in the surgical cavity (TBR 2.2 Figure 3, panel a-c). Histopathological analysis of the biopsy showed squamous cell carcinoma, however there was no direct correlation with the location of the tumor-positive resection margin at the excised specimen. The tumor-positive biopsy is therefore diagnosed as in-transit metastasis. Despite adjuvant radiotherapy (cumulative dose 70Gy) without chemotherapy due to severe co-morbidity, the patient developed a recurrent tumor during radiotherapy located at the deep tongue musculature. During tumor resection of *patient 9*, fluorescence imaging of the medial side of the surgical area showed a fluorescence-positive lesion (TBR 6.2, Figure 3, panel d-f). The biopsy of this lesion showed severe dysplasia without invasive carcinoma. Histopathological analysis of the surgical specimen revealed a tumor-positive surgical margin at the medial resection plane along with the presence of high-grade dysplasia, which correlated with the location of the fluorescent lesion in the surgical cavity. Lastly, the surgical cavity of *patient 10* showed a large area of fluorescence (TBR 5.7, panel g-i). Based on clinical arguments, related to the extent of the surgical defect, no biopsy was performed. The *ex vivo* fluorescence correlated with a focal tumor-positive surgical margin which corresponded to the location of the fluorescence in the surgical cavity (Supplemental Figure S2, panel q-t). In one case (patient 3), a false-positive fluorescence signal was observed in the surgical cavity (Supplemental Figure S4). Here, the lesion was suspected for perineural growth, however the histopathology showed no tumor involvement nor any other reason for aspecific ONM-100 activation.

Realtime in vivo analysis

Figure 3 | Real-time in vivo HNSCC analysis

Patient 7 with an in-transit metastasis detected after biopsy of a fluorescent spot in the surgical cavity (a-c). Patient 9 with a tumor in the mandible with a positive spot for fluorescence in the surgical cavity, which showed high-grade dysplasia without invasive carcinoma (d-f). Patient 10 with a tumor in the mandible with a clear fluorescent spot indicating a focal tumor-positive surgical margin as confirmed by histopathological assessment (g-i). Mean Fluorescence Intensity (MFI) of the fluorescent spot compared to the background for all three patients (j) and Tumor-to-Background-Ratios derived from the MFI data in all three patients (k).

Real-time surgical margin determination – defining the room for improvement

Three patients (patient 9, 11, 12) underwent an additional re-resection within a month after initial surgery according to SOC due to a tumor-positive or close surgical margin. During initial surgery of all three cases, the excised specimen was diagnosed as fluorescence-positive. Interestingly, during fluorescence imaging of the surgical cavity, no suspected lesions were detected in two of these cases (patient 11 and 12). This is congruent with the histopathological findings, showing a tumor-positive surgical margin (<1mm), but no cut-through of the tumor, thus tumor cells within 0-1mm of the border. As a result, histopathological analyses after the surgical re-resection in a second surgery showed no remaining tumor cells in these two HNSCC (re-)resected specimens. The remaining patients diagnosed with a tumor-positive or close resection margin positive for fluorescence (patient 2, 3, 4, 5, 7, 10) received no additional surgery based on clinical considerations such as severe comorbidity as decided within the multidisciplinary tumor board.

DISCUSSION

The field of fluorescence-guided surgery in oncology is expanding rapidly, but standardized methods to evaluate imaging results are not widely used. Our results indicate that a standardized *ex vivo* fluorescence-guided imaging method for margin assessment using the pH-activated imaging agent ONM-100 shows substantial clinical potential for real-time intra-operative decision making. We identified all tumor-positive margins by detecting fluorescent lesions at the surgical resection margin with a tumor- to-background ratio > 1.5 . This easy implementable framework, which can be implemented in the surgical theatre, can be used immediately after surgical excision allowing an alteration of the surgical strategy during initial surgery. Subsequently, we showed that ONM-100 can detect remaining tumor and severe dysplasia in the surgical cavity which was otherwise missed during standard of care surgery.

We suggest the use of our standardized fluorescence framework, combined with ONM-100, for *real-time* surgical margin assessment in HNSCC and surgery (Figure 4). Briefly, when a fluorescent lesion at one of the resection margins is detected (threshold TBR > 1.5), and if anatomical borders allow an additional resection *in situ*, an immediate re-resection can be considered.¹² This imaging procedure can be performed in the surgical theatre to improve surgical outcome. After tumor excision, imaging of the surgical cavity is performed subsequently to fluorescence imaging of the freshly excised surgical specimen which can guide the surgeon in performing an immediate re- resection. Additionally, fluorescence imaging of the surgical cavity can detect occult tumor and/or dysplastic lesions, whether or not related to the primary tumor, as highlighted by the observations in our cohort, in which one occult additional tumor lesion and a dysplastic lesion were found using fluorescence imaging of the surgical cavity. We show that FGS using ONM-100 could guide the surgeon to perform an immediate re-resection and potentially prevent (unnecessary) additional re-resections or adjuvant treatment. This potential is underlined by the fact that after an initial tumor- positive surgical margin two HNSCC re-resections in this series were negative for residual tumor. In the unfortunate event that it is not possible to perform an additional resection, which accounts not only for head- and neck cancer patients, the surgeon receives immediately feedback on the clinical situation and is able to alter the surgical plan (i.e. no further surgery, marking of positive spot for post-operative radiotherapy).

Figure 4 | Flowchart real-time clinical decision making

Suggested flowchart for fluorescence-guided surgery and surgical decision-making using fluorescence with this tumor generic fluorescence imaging agent. An additional surgical resection can be made in different scenarios when fluorescent spots with a Tumor-to-Background Ratio of >1.5 is observed on either the surgical specimen or in the surgical cavity.

In the current study, intra-operative *ex vivo* fluorescence imaging for margin assessment has been prioritized above *in vivo* fluorescence imaging. *Ex vivo* specimen imaging using a closed-field and standardized imaging device allows for standardization of measurements, which is highly relevant in fluorescence-guided imaging where multiple factors like distance to the camera, angle of illumination and environmental light can influence imaging results severely.¹³ With the availability of a close-field imaging device at the surgical theatre, tissue can be imaged immediately after surgical excision allowing for direct surgical decision making, as also has been described by other groups.^{10,14,15} As the close-field imaging device used in the current study has a limited field of view, future developments in field of view, resolution and imaging quality might further enhance intra-operative clinical decision-making.

Despite the investigated imaging agents showed promising results in previous studies, visual discrimination between tumor and non-tumor tissue remains challenging due to high, a-specific background signals in non-tumor tissue.^{5,7,8,11} A low background signal, a consequence of the design of ONM-100, shows a clear and sharply delineated fluorescent signal in tumors in this study. This discriminative strength of ONM-100 is illustrated by the fact that the tumor-positive resection margins were clearly highlighted compared to the non-involved adjacent resection margin. Moreover, ONM-100 was administered within 24 hours of surgery, providing major benefits over other fluorescence imaging agents which are administered 2-5 days prior to surgery.^{5,7,16} Indeed, we have seen some false-positive fluorescence signal in salivary gland tissue, as is previously described as well.⁵ The exact reason for this activation needs to be further analyzed in future studies, however, as we believe that a surgeon is able to differentiate salivary gland tissue from tumor tissue, we believe this might not be a significant clinical problem.

Based on our experience as a tertiary referral hospital, performing complex surgery often in late-stage HNSCC disease (Supplemental Figure S3), we identified three possible scenarios originating from the findings in this study following the detection of a fluorescent lesion. First, fluorescent lesions can be resected with an adequate margin without compromising vital structures. Second, an identified fluorescent lesion cannot be resected due to anatomical borders or the extent of disease. Third, new unidentified lesions not directly related to the initial tumor or irresectable lesions are identified which might change or cease the surgical plan. The first and last scenario occurred in this particular study. As this study shows, the fluorescent lesions detected at the excised specimen correlated in 100% of the cases to tumor-positive resection margin and in 60% of the cases of to close resection margins. This implicates that, in theory, in ten patients an immediate re-resection could be considered, thereby preventing a second surgery. This is highly relevant, since not all patients were eligible for second surgery due to their intrinsic comorbidities. From the possible ten re-resections, one re-resection would result in a case of overtreatment due to a false positive fluorescent signal according to the current guidelines on tumor involved margins. If this eventually would lead to a higher local tumor control remains to be studied. We assume that in case of a successful immediate re-resection at the primary tumor site, adjuvant therapy can at least be de-intensified from the concomitant combination of chemoradiation to adjuvant radiotherapy alone. We therefore consider these data encouraging for further exploring the use of ONM-100 for the purpose of intra-operative margin assessment in a larger phase II trial.

Recent studies in HNSCC suggest a cutoff point of 2 mm between a tumor-positive and a tumor-negative surgical margin, as > 2 mm margin shows no significant increase in disease-specific survival.^{3,17} Therefore, a method that could discriminate between tumor presence within 2mm and > 2 mm of the surgical border would be suited for *real-time* surgical guidance in HNSCC patients. Interestingly, all fluorescence-negative surgical margins found in this study had a surgical margin of at least > 2 mm, again underlining the clinical applicability of this technique. It should be noted that the numbers of tumor-positive margins detected in the current study are outside the average norm.¹⁸ However, a tertiary referral hospital (UMCG) receives delicate and late-stage disease patients which a high a priori chance for tumor-positive surgical margins.

In conclusion, this study demonstrates the potential added value of specimen-driven fluorescence imaging of the surgical specimen in HNSCC patients after administration of the optical imaging agent ONM-100. We found a 100% sensitivity for tumor-positive surgical margin detection using this technique and showed the potential for the intra- operative detection of occult disease. ONM-100 is easy to implement into standard clinical care due to the clear visual discrimination between tumor and non-tumor tissue, therefore having great potential in assisting in clinical decision making during HNSCC surgery, preventing under- and over-treatment.

SUPPLEMENTAL DATA

Standardized fluorescence guided decision

Supplemental Figure S1 | Real-time clinical decision making framework

ONM-100 was administered intravenously approximately 24h ($\pm 8h$) hours prior to surgery. (2) Fluorescence-guided imaging of the surgical specimen was performed ex vivo directly after excision of the tumor using a closed-field imaging device for positive margin detection (3) fluorescence-guided imaging in vivo of the surgical cavity was performed for detection of positive margins and/or occult disease (4) During all phases of standard pathology processing, fluorescent images of the whole specimen and fresh tissue slices were obtained (5) Correlation of the fluorescent signal on whole specimen and with standard histopathology was performed based on H/E slices by a board-certified pathologist blinded for fluorescence.

Supplemental Figure S2 | Tumor-positive head- and neck cancer surgical margins

All five tumor-positive surgical margins imaged directly after tumor excision with the PEARL Trilogy are depicted. A region of interest (ROI) was drawn around the fluorescent spot and of the remaining tissue of the whole surgical specimen (denominated as background) and the Mean Fluorescent Intensity (MFI) was calculated. TBR was calculated as $MFI_{\text{fluorescent spot}} / MFI_{\text{background}}$. The ROI of the fluorescent spot was correlated to histopathological assessment. All tumor-to-background ratios of the five patients with a tumor-positive surgical margin. (u)

Supplemental Figure S3 | Clinical imaging of late-stage HNSCC disease
Representative examples of pre-operative imaging of HNSCC tumors included in the current study.

Supplemental Figure S4 | False positive intra-operative lesion
Fluorescence imaging of the surgical cavity of patient 3, illustrating the false positive lesion in the surgical cavity as described in the manuscript.

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CHAPTER 5

Improving visualization of colorectal metastatic cancer using laparoscopic fluorescence-guided surgery

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ABSTRACT

Extensive surgical treatment for peritoneal metastases originating from colorectal cancer provokes high morbidity and perioperative mortality rates, therefore careful and objective patient selection prior to such a procedure is crucial. Tumor-specific fluorescence-guided laparoscopy is a potential imaging technique for the improvement of patient selection. The aim is to select the patient that benefits the most of extensive and valuable surgical treatment, in terms of disease-free survival, overall survival and quality of life. Cancer-upregulated proteins and tumor-specific biological processes with matching fluorescence imaging agents could guide the surgeon to improved identification of malignant tissue during diagnostic laparoscopy and for detection of non-visible small tumor lesions during cytoreductive surgery.

INTRODUCTION

Colorectal cancer (CRC) is one of the most commonly diagnosed cancer types with a worldwide incidence of 1.8 million patients. Peritoneal metastases (PM) used to be a form of end-stage disease with an incidence differing between 4 to 40% worldwide.^{1,2} PM is defined as the presence of metastatic tumor nodules spread over the peritoneal surface throughout the abdominal cavity and can arise from colorectal cancer, gynaecological cancers and peritoneal cancers.³ Over the last two decades, performing cytoreductive surgery (CRS) with hyperthermic intraperitoneal chemotherapy (HIPEC) has improved overall survival (OS) shifting PM treatment from a palliative setting - using systemic chemotherapy - towards a potential curative treatment option depending on the preoperative condition of the patients and tumor load determined by the peritoneal carcinomatosis index (PCI).⁴ In adequate selected patients, CRS combined with HIPEC to reduce microscopic malignant disease is performed. CRS combined with HIPEC improves OS from 21 to 63 months and increases the five-year survival rate up to 40% compared to palliative chemotherapy.^{5,6} With a complete cytoreduction, mortality rate due to recurrence of disease decreases with 39% when compared to non-complete cytoreduction.⁷ Despite promising initial results, this complex and extensive surgical treatment introduces a high risk of procedure-related mortality (0-8%), morbidity (12-68%) and extensive postoperative rehabilitation with prolonged hospitalization.⁸ In 25% of all planned patients the initial surgical procedure is terminated prematurely due to excessive presence of irresectable tumor or high PCI, also referred to as an "open and close procedure (OC procedure)". Moreover, 30% of patients develop recurrence of disease within one year.⁹ These data illustrate the impactful consequences of this extensive treatment for our peritoneal metastasized patients and the need for technical improvement to identify the best patients for this extensive surgical treatment.

During the surgical treatment of PM originating from CRC there is a clear clinical need for introduction of innovative techniques to improve patient selection and eventually patient outcome. A more advanced, intra-operative imaging and diagnostic selection tool has the potential to create more tumor-specific and precise cancer surgery. The introduction of fluorescence-guided surgery (FGS) has demonstrated the feasibility to identify extra PM lesions during open surgery and could enable introduction of fluorescence-guided laparoscopy for more adequate patient selection and improvement of the treatment regimen and outcomes.¹⁰⁻¹²

Spread of peritoneal deposits

PM pathogenesis can be explained by three different models: A) dissemination from the primary tumor, B) primary peritoneal tumor or C) peritoneal deposits with an independent origin. The most frequent form of dissemination is exfoliation of malignant cancer cells, when the tumor has expanded through the serosa. Subsequently, there are two different pathways for the attachment of the malignant cells to the peritoneum. In the first pathway, in absent or rounded (cuboidal) mesothelial cells, the integrins facilitate the attachment of the malignant cancer cells to the submesothelial connective tissue creating peritoneal deposits. In the second pathway, loose malignant cells adhere to mesothelium directly through adhesion molecules.¹³

Surgical standard

The current surgical strategy in patients with colorectal PM is initiated by efforts to improve patient selection with a white light diagnostic laparoscopy (WL-DLS) to visualize the extent of disease by scoring the PCI.¹⁴ A higher PCI indicates more tumor load and a lower 4-year survival rate.⁷ WL-DLS is safe and reduces the amount of OC procedures with 20-40%.¹⁵ During WL-DLS surgeons obviously depend on visual inspection alone without tactile information, and therefore adequate identification of malignant compared to benign tissue remains extremely challenging. The presence of benign scar tissue originating from neoadjuvant treatment and previous surgery makes adequate identification complex during WL-DLS and CRS. Consequently, small tumor lesions may be easily missed and clinically suspicious lesions could therefore be benign.

Fluorescence-guided tumor visualization

In the last decade, FGS using optical imaging and non-ionizing tumor-specific fluorescence imaging agents has emerged as an innovative real-time imaging technique to aid surgeons for the enhancement of intra-operative tumor visualization and increasing margin assessment in oncological surgery.¹⁶⁻¹⁸ FGS as such serves as a 'red-flag' imaging technique to assist in visualization of small tumors, peritoneal deposits and leads to adequate differentiation between benign and malignant tissue. Various FGS studies showed an increase of real-time and adequate tumor-positive margin detection during surgery in a variety of tumors, potentially increasing surgical quality using FGS.¹⁸⁻²⁰ Fluorescence-guided surgery uses optical properties of non-ionizing endo- and exogenous photons to visualize oncological phenotype and is relatively easy to implement during surgery. The near-infrared (NIR) spectrum (650-900 nm), invisible for the human eye, is used as NIR light can travel millimeters up to centimeters through tissue allowing for visualization of tumor-(non)specific fluorescence imaging agents. Different exogenous non-targeted or targeted contrast agents are described, where the targeted imaging agents contain a targeting moiety and a signal agent using fluorophores. Special imaging devices, like open fluorescence cameras, are used to visualize the imaging agent and therefore biological information of tumor tissue and benign tissue. Moreover, fluorescence imaging has become available in commercial laparoscopes, calibrated to adequately detect tumor non-specific indocyanine green (ICG, excitation 780 nm).²¹ However, for the detection of low-dose tumor-specific imaging agents, most commercially available imaging systems are not sensitive enough for adequate detection. Fluorescence imaging agents have relatively low fluorophore concentrations *in vivo* as conjugation with a tumor-specific component and human dose restrictions hinder increased fluorophore concentration. Moreover, imaging agent distribution varies in between tumor types, tumor size and patient characteristics.

Improving peritoneal metastases visualization

Intra-operative imaging of colorectal PM using ICG has been performed, showing the potential of FGS for enhanced tumor visualization.²² As ICG is not tumor-specific, a major drawback is the lack of sensitivity and specificity, which is extremely important during WL-DLS for adequate patient selection based on true-positive and true-negative imaging of suspected lesions.

A variety of studies investigated tumor-specific fluorescence imaging agents against cell surface receptors using intra-operative and *ex vivo* fluorescence imaging. Cancer-up-regulated proteins, with an enhanced expression on tumor cells, like Endothelial Growth Factor Receptor (EGFR), Vascular Endothelial Growth Factor (VEGF) and Carcinoma-Embryonic Antigen (CEA) have been widely used as molecular target for FGS.^{11,18,20,23} For PM, a variety of potential targets were identified for intra-operative imaging, including VEGF-A and CEA.²⁴ VEGF-A, involved in tumor-induced angiogenesis, is upregulated in 93% of colorectal PM.²⁵ An improved surgical outcome and survival using fluorescence-guided laparoscopy has been shown in mouse models of human pancreatic and colon cancer.²⁶ In our centre, the VEGF-A targeted Bevacizumab (Avastin®, Roche) conjugated to the NIR fluorescent agent IRDye-800CW (LI-COR Biosciences, Lincoln, NE, USA) is used in a variety of clinical trials. This fluorophore-labeled therapeutic monoclonal antibody showed adequate detection of intra-abdominal submillimeter malignant tissue in mouse models.²⁷ After IRB approval, bevacizumab-800CW has shown to be safe in all dosing groups (4.5 to 50 milligrams) and is administered in patients intravenously two or three days prior to cancer surgery and showing promising results in the detection of breast cancer, sarcomas and oesophageal carcinoma.^{28,29} In 2016, Harlaar *et al.* showed the clinical feasibility of bevacizumab-800CW in seven patients with colorectal PM undergoing an explorative laparotomy as part of CRS+HIPEC.¹⁰ Eighty peritoneal areas were imaged using an open fluorescence intra-operative camera system (SurgVision B.V., Groningen, The Netherlands). All 29 non-fluorescent resected areas proved to be benign on final histopathology, thus potentially indicating a sensitivity of 100%. On the other hand, in 27 out of 57 fluorescent resected areas (47%) from the fresh surgical specimen, tumor tissue was identified. These results show the potential for detecting peritoneal deposits using bevacizumab-800CW also during WL-DLS, which could help to assess the true extent of peritoneal disease more accurately and to prevent performing CRS+HIPEC surgery in patients who will not benefit from this complex abdominal procedure in terms of survival and quality of life.

Other colleagues from Leiden University showed the safety and feasibility of SGM-101, an anti-CEA monoclonal antibody, during open surgery in patients with colorectal PM. Fluorescence-positive malignant lymph nodes, both superficially and deeper seated, which were missed during standard surgery were visualized changing the surgical strategy in one out of three patients. During a follow-up study, additional lesions were observed using SGM-101 during laparotomy mostly increasing fluorescence PCI. FGS improved macroscopic cytoreduction and thus potentially improves surgical quality, and in later stage overall and disease-free survival for patients.¹¹ Based on both clinical studies, we conclude that FGS using monoclonal antibodies could be used during WL-DLS for adequate patient selection for CRS+HIPEC and during CRS+HIPEC for adequate surgical cytoreduction. A recent study showed the possibility of visualization of PM and colorectal cancer targeting tumor acidosis with a smart-activatable pH-activated nanoprobe, showing the potential for dual use of this generic imaging agent and the potential of using a variety of imaging agents and fluorophores for PM detection (table 1).¹⁹

Table 1 | Clinical available fluorescence imaging agents for PM visualization

GMP available imaging agent	Description	Excitation/emission wavelength
Bevacizumab-8oCW ¹⁰	VEGF targeting antibody with IRDye800. Administration 2-3 days prior to surgery	778 / 794 nm
SGM-101 ^{11,12}	Monoclonal antibody against CEA. Administration 4 days prior to surgery	686 / 704 nm
ONM-101 ¹⁹	pH-sensitive micelles with ICG. Administration 1 day prior to surgery	780 / 820 nm

Optimizing surgical treatment

In order to optimize surgical strategy in this complex patient group, we aim to perform a feasibility WL/fluorescence-guided-DLS study using bevacizumab-800CW in patients with colorectal PM. The ability of laparoscopic fluorescence imaging to detect extra fluorescence-positive PM, as has been shown using bevacizumab-800CW and SGM-101 before, could potentially change clinical decision making (Figure 1). The first aim is to identify the optimal dosage for laparoscopic imaging and to compare fluorescence PCI to clinical PCI during WL-DLS. In the future, a combination of fluorescence WL-DLS and fluorescence imaging during CRS+HIPEC could be performed to compare both fluorescence imaging methods and their effects in the same patient group.

Figure 1 | Proposed surgical workflow

CONCLUSION

Adequate selection of eligible patients with colorectal PM for CRS+HIPEC remains difficult. Improving patient selection and surgical quality before undergoing CRS+HIPEC is clinically relevant due to high morbidity and perioperative mortality rates of this intentionally curative treatment. FGS using tumor-specific imaging agents targeting cancer-upregulated proteins has proven its feasibility to delineate malignant tissue during laparotomy and thus has the potential to increase intra-operative detection of colorectal PM during laparoscopy. However, those studies are performed in relatively small numbers of patients. Further expansion in phase II validation studies is needed to confirm those primary results during laparotomy and laparoscopy. We propose to perform a phase II clinical study targeting VEGF-A with bevacizumab-800CW to improve intra-operative detection using fluorescence-guided laparoscopy.

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CHAPTER 6

Fluorescence imaging of angiogenesis ex vivo in (non)-culprit human carotid atherosclerotic plaques using bevacizumab-800CW

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ABSTRACT

Vascular endothelial growth factor-A (VEGF-A) is assumed to play a crucial role in the development and rupture of vulnerable plaques in the atherosclerotic process. We used a VEGF-A targeted fluorescent antibody (bevacizumab-IRDye800CW [bevacizumab-800CW]) to image and visualize the distribution of VEGF-A in (non-)culprit carotid plaques *ex vivo*. Freshly endarterectomized human plaques ($n = 15$) were incubated in bevacizumab-800CW *ex vivo*. Subsequent NIRF imaging showed a more intense fluorescent signal in the culprit plaques ($n = 11$) than in the non-culprit plaques ($n = 3$). A plaque received from an asymptomatic patient showed pathologic features similar to the culprit plaques. Cross-correlation with VEGF-A immunohistochemistry showed co-localization of VEGF-A overexpression in 91% of the fluorescent culprit plaques, while no VEGF-A expression was found in the non-culprit plaques ($p < 0.0001$). VEGF-A expression was co-localized with CD34, a marker for angiogenesis ($p < 0.001$). *Ex vivo* near-infrared fluorescence (NIRF) imaging by incubation with bevacizumab-800CW shows promise for visualizing VEGF-A overexpression in culprit atherosclerotic plaques *in vivo*.

INTRODUCTION

Carotid artery atherosclerosis is a leading cause for ischemic stroke, accounting for approximately one in four stroke cases.¹ The annual stroke risk due to carotid atherosclerosis is increased by 10-20% compared to patients without carotid artery stenosis.² A study based on the large population-based Rotterdam cohort study demonstrated that 80% of the cohort over 55 years of age had some degree of carotid artery atherosclerosis.³ Risk factors for the development of atherosclerosis include hypertension, dyslipidemia, diabetes mellitus, obesity and smoking. Most of these risk factors are increasingly prevalent amongst the world population as the result of ageing and the earlier emergence of comorbidities.⁴

Formation of an atherosclerotic plaque within the vessel wall can cause partial or total vessel occlusion. Plaque rupture inevitably causes downstream ischemic damage in other organs.⁵ Currently, there is no imaging modality available to stratify the risk of rupture *in vivo*. This leads to unnecessary surgical intervention in many patients, which is not desirable as the mortality rate is approximately 3% for this type of operation.⁶ Identifying intraplaque biomarkers might possibly reveal the extent of plaque (in)stability and therefore aid in risk-stratification. Besides the composition and volume of the plaque, pathological plaque properties are crucial in assessing its stability. These properties include inflammation, lipid accumulation, angiogenesis, calcium precipitation, proteolysis, apoptosis, and thrombosis.^{5,7,8} Ultimately, a combination of increased endothelial shear stress and increased tensile (wall) stress facilitates rupture of the unstable plaque.⁹ Specific molecular imaging techniques can aid in predicting the risk of stroke by visualizing and quantifying plaque characteristics. Currently, carotid endarterectomy (CEA) is primarily performed on eligible patients with carotid stenotic lesions and signs of transient ischemic attacks or minor ischemic stroke to reduce the risk of future events. Imaging techniques might enable reliable selection for CEA surgery before symptom onset, in for example a high-risk patient population.

Positron emission tomography (PET) and single-photon emitted computed tomography (SPECT) have been investigated in detecting the vulnerable plaque. Although a recent meta-analysis found that ¹⁸F-FDG uptake was associated with inflammation in the symptomatic plaque, a clear cut-off point for the vulnerable plaque could not be established due to the heterogeneity of data previously described.¹⁰ Another study reported that ¹⁸F-FDG uptake in recent transient ischemic attack (TIA) or amaurosis fugax patients did not differ significantly from asymptomatic patients, rendering it a questionable clinical indicator for plaque vulnerability.^{11, 18} F-sodium fluoride was investigated as a tracer for plaque calcification in both myocardial infarction and symptomatic carotid stenosis patients. The former observed uptake in almost all culprit plaques, but also in half of their non-culprit plaque group (stable angina pectoris patients).¹² The latter did not observe a statistical difference between uptake of culprit and non-culprit carotid plaques, based on time interval between symptom onset and CEA.¹³

Ultrasound techniques have also been investigated in atherosclerotic plaques. Plaque surface irregularity was also investigated as risk indicator for cerebrovascular events. Kanber *et al.* demonstrated an accuracy of 83% in symptomatic patients with ultrasound imaging of plaque surface irregularities in combination with the degree of stenosis, whereas plaques from asymptomatic patients have a significantly smoother surface.¹⁴ However, it is unclear whether plaque surface irregularities were true plaque ulcerations or other, incidental, surface defects. Additionally, identification of irregularities depends on scanning resolution and might be missed due to two-dimensional ultrasound imaging.

A non-invasive clinical follow-up study was performed in patients who underwent carotid endarterectomy, collecting test and imaging results from the hospitals concerned. During this study, a yearly risk assessment was performed based on analysis of the endarterectomized samples and the clinical patients characteristics, demonstrating that plaque characteristics such as macrophage infiltration and lipid core extent alone were not predictive of future cardiovascular events due to other atherosclerotic plaques.¹⁵ A potential new target for plaque categorization is intraplaque angiogenesis.^{15–17} Angiogenesis within the atherosclerotic plaque is considered to arise from initial inflammation of the arterial wall and subsequent influx of macrophages resulting in a relative localized hypoxia, the latter being the most important driver through expression of HIF-1 α for vascular endothelial growth factor-A (VEGF-A) expression.^{18,19} Consequently, intraplaque release of VEGF-A is stimulated, prompting the formation of immature, leaky blood vessels prone to rupture causing hemorrhage and thrombus formation, which results in plaque instability.^{16,18,20} The Athero-Express study demonstrated plaque neovascularization and plaque hemorrhage to be significantly related to adverse cardiovascular outcome.²¹ Previously, Shah *et al.* demonstrated the use of contrast-enhanced ultrasound imaging to detect neovascularization in carotid atherosclerotic plaques and found a moderate correlation with histopathological staining.²² Practical implication remains a challenge, however, due to lack of an optimal enhancement quantification method and standardized protocols.²³ Accurate detection of the angiogenic process, and thus progression towards a vulnerable plaque, might contribute to the identification of high-risk patients.

Conventional current imaging techniques possess a central role in visualizing the atherosclerotic plaque, such as Computed Tomography Angiography (CT-A) and duplex ultrasound (US) as part of the standard of care.²⁴ Relatively new in the (clinical) cardiovascular field is near-infrared fluorescence (NIRF) imaging. This modality uses fluorophores as imaging compound instead of the generally used radioactive imaging agents. *In* and *ex vivo* near-infrared fluorescent (NIRF) imaging can provide real-time information about molecular tissue composition with high sensitivity.^{25,26} In order to image angiogenesis, previously a VEGF-A targeted antibody (bevacizumab) has been conjugated to a NIR fluorescent dye (IRDye800CW) for successful fluorescent imaging in solid tumors (i.e. breast cancer, peritoneal carcinomatosis, esophageal lesions and colorectal adenomas).^{27–30} Bevacizumab-800CW showed first of all to be safe, to have a high sensitivity and specificity, and allowed for accurate and reproducible imaging of

angiogenesis. Previously, our research group has shown that radiolabeled ^{89}Zr -bevacizumab is an effective and specific method for the detection of VEGF-A in carotid endarterectomy specimens.³¹ The advantage of bevacizumab-800CW over ^{89}Zr -bevacizumab is that the former has no radiation burden and is relatively less expensive than the latter. In this study, the feasibility of bevacizumab-800CW-based optical imaging for the detection of carotid plaque instability was assessed. The aim of this study was to determine whether the optical tracer bevacizumab-800CW is suitable for *ex vivo* visualization of intraplaque angiogenesis by means of identifying VEGF-A presence, and thereby able to distinguish between culprit and non-culprit atherosclerotic plaques.

METHODS

Study design

For this *ex vivo* feasibility study, fresh human carotid specimens were obtained from patients undergoing carotid endarterectomy between September 2017 and April 2018 in the University Medical Center Groningen (UMCG). Previous literature demonstrated that 'positive' plaques corresponded to the patients with recent events (i.e. culprit plaques) and 'negative' plaques to patients with a remote history of ischemic stroke (i.e. non-culprit plaques).³² Odds ratios of present plaque characteristics as risk indicators for stroke were demonstrated to be significantly greater for plaques retrieved from patients operated within 30 days after the ischemic event than in plaques retrieved after 30 days after symptom onset. For high microvessel density, these odds ratios were 4.52 and 1.47, respectively.³² In this study, we adopted this dichotomy: 'positive' culprit plaques were defined as plaques in patients with recent events (i.e. cerebrovascular accident, transient ischemic attack, amaurosis fugax), and 'negative' non-culprit plaques were defined as plaques in patients with no events in the last four weeks. For all patients, clinical and demographic data were collected from medical records, using the hospital's electronic medical record system, including medication use and cardiovascular risk factors that were present before symptom onset. The study was released by the Medical Ethical Committee of the UMCG and is in accordance with the Helsinki Declaration (2013). It was decided that patient informed consent was not required as the study uses waste tissue-material, that is not restricted by the Dutch WMO (Wet Mensgebonden Onderzoek, which translates to 'law on research involving human subjects'). The IRB of the UMCG approved this study. For all patients, it was checked if objection statements about the use of waste material were ever recorded by the hospital. Data analysis was performed anonymously. Primary endpoints were the accumulation of *ex vivo* incubated bevacizumab-800CW in carotid plaques by fluorescence macro- and microscopy, and its correlation with VEGF-A expression, macrophage infiltration (CD68), microvessel density (CD34), microcalcifications (Von Kossa) and structural integrity of the vessel wall (smooth muscle actin; SMA), determined by semi-quantitative analysis of (immuno)histochemical stainings. For these parameters, culprit and non-culprit plaques were compared.

Fluorescence imaging techniques

Bevacizumab (Avastin; Genentech, South San Francisco CA, USA) conjugation with the fluorescent dye IRDye800CW was performed at the department of Clinical Pharmacy and Pharmacology, as described earlier.³³ An optimal dose of 1 µg/ml was determined by submersing the specimens in various solutions (4 µg/ml, 2 µg/ml, and 1 µg/ml bevacizumab-800CW in PBS) for one hour in a dark room to prevent photobleaching. The used concentrations were based on theoretical blood concentrations after intravenous administration of 4.5 mg, 10 mg, and 25 mg bevacizumab-800CW, respectively. The imaging procedures with the optimal dose were as follows. Immediately after excision, the specimens were rinsed with PBS. Subsequently, images of the whole tissue were acquired with the PEARL imager (LI-COR Biosciences, Lincoln NE, USA). The specimens were then submersed in a 1 µg/ml bevacizumab-800CW solution for one hour at room temperature. PEARL images were performed of the whole tissue specimen, after which hot spots (i.e. spots with 95% of the highest fluorescent signal (expressed in Mean Fluorescence Intensity [MFI])) were selected. The MFI was only calculated from the subsequent PEARL images. All images of all specimens were scaled to the same intensity. The spots were formalin-fixed, paraffin-embedded (FFPE) and cut in 3 µm sections.

The FFPE blocks were analyzed with the Odyssey fluorescent flatbed scanner (LI-COR Biosciences, Lincoln NE, USA). In order to cross-correlate a fluorescent signal within the corresponding 3 µm slides with additional histopathological staining, the sections were deparaffinized (10 min xylene) and analyzed with the Odyssey scanner. Afterwards, these 3 µm slides were stained with hematoxylin and eosin (H&E) for cell morphology and to determine what part of the plaque displayed fluorescent signal.

In additional consecutive 3 µm slides cell nuclei were visualized with Hoechst staining (Invitrogen, Carlsbad CA, USA) in the same manner as we described previously.²⁹ Leica NIR fluorescence microscopy was carried out to colocalize bevacizumab-800CW and VEGF-A. For image processing and analysis, LAS-AF software (Leica Microsystems, Wetzlar, Germany) and Fiji/ImageJ (version 1.8.0., LOCI, University of Wisconsin, USA) were used as described previously.

(Immuno)histochemistry

To allow for examination of the pathological status (i.e. vulnerability) of the plaques, for each spot slides were double stained for VEGF-A and either Von Kossa, CD34, CD68 or SMA. The latter three immunohistochemical stainings were performed by standardized machine protocols. For the staining of microvessels, slides were incubated with anti-CD34 (mouse IgG₁ κ monoclonal, Dako, clone QBEnd/10). Slides were incubated with anti-CD68 (mouse IgG₃ κ monoclonal, Dako, clone PG-M1) for the staining of macrophages. Smooth muscle actin in the vessel walls was stained with anti-SMA (mouse monoclonal, Dako, clone 1A4). All slides were incubated for one hour at room temperature. Consecutively, the slides were incubated with horseradish peroxidase (HRP) secondary antibodies, and afterwards chromogenic staining with 3,3'-diaminobenzidine (DAB)

was performed for color development. Hematoxylin was used to counterstain the slides. For VEGF-A, slides were deparaffinized and rehydrated with xylene (10 min) and graded alcohols. For heat-induced antigen retrieval (15 min), 100 mM Tris/EDTA buffer (pH 9.0) was used. Endogenous peroxidase was blocked for 30 min with 30% hydrogen peroxide, followed by avidin-biotin blocking. Subsequently, the slides were incubated overnight at 4°C with a polyclonal rabbit anti-human VEGF-A antibody (RB9031, Thermo Fisher Scientific, Waltham, MA, USA). Slides were incubated with goat anti-rabbit biotin in phosphate-buffered saline with 1% bovine serum albumin [PBS 1%BSA] with 1% AB serum, and consecutively with alkaline-phosphatase conjugated streptavidin in PBS 1% BSA with 1% AB serum, both for 30 min. For color development, alkaline-phosphatase activity was visualized using naphthol AS-MX phosphate and Fast Blue salt (30 min). The slides were washed with demineralized water and cover slipped with Kaiser's glycerin. PBS was used for washing, and all steps were performed at room temperature unless indicated otherwise.

For the Von Kossa staining, the slides were first submersed with 1% silver nitrate solution for 60 min in broad daylight, followed by 3% sodium thiosulfate (5 min). The slides were counterstained with nuclear fast red (3 min), after which dehydration was performed with graded alcohols. The slides were then air-dried (30 min) and cover slipped with mounting medium. Demineralized water was used throughout washing.

The stained slides were digitized with the NanoZoomer Digital Pathology Scanner (Hamamatsu Photonics K.K., Hamamatsu City, Japan). The imaged slides were then analyzed and evaluated with Aperio ePathology (Leica Biosystems, Wetzlar, Germany) software. To score the degree of VEGF staining, the H-score was calculated in the same manner as described previously by our group³⁴, determined by semi-quantitative combined assessment of the percentage of stained cells and the staining intensity (0-300; continuous scale; 0-100: negative/weak; 101-200: moderate; 201-300: strong). Von Kossa, CD34, CD68 and SMA stainings were categorized semi-quantitatively (score 1 = weak; 2 = moderate; 3 = strong). The pattern of fluorescent signal was compared to corresponding VEGF-A-stained slides by fitting the VEGF-A region of interest (ROI) on the corresponding fluorescence image and determining the extent of overlap between VEGF-A expression and fluorescent signal.

Data and statistical analysis

Data were analyzed with SPSS Statistics 24 (IBM, Armonk, NY, USA) and Fiji/ImageJ (version 1.8.0., LOCI, University of Wisconsin, USA). Regions of interest (ROIs) were drawn based on the VEGF-A staining (95% of the strongest expression, determined by standardized counting algorithms). The mean fluorescence intensity of this ROI was measured quantitatively. Categorical data was tested with Chi-Square association tests. A p -value ≤ 0.05 was considered to be statistically significant.

RESULTS

Patient characteristics

Carotid artery plaque specimens from 17 patients were included in the study ($n = 17$), of which three were used for dose-finding analysis. The optimal dose was used in 15 plaques: 11 plaques in the culprit group and three in the non-culprit group, based on the period of time between symptom onset and carotid endarterectomy (CEA), and one plaque was retrieved from an asymptomatic patient and was analyzed separately. Patient characteristics of the culprit and non-culprit groups are shown in Table 1. Specimens of the common, internal and external carotid artery were used. Of all patients, six suffered from a cerebrovascular accident (CVA), six had a transient ischemic attack (TIA), two suffered from amaurosis fugax, and one patient was asymptomatic but eligible for elective carotid endarterectomy. The time interval between symptom onset and CEA was on average 2 weeks (range: 2 – 4 weeks) for the culprit group and 7 weeks (range: 5 – 8 weeks) for the non-culprit group. Some patients did not undergo surgery within the standard of care 2 weeks after symptom onset, caused by either patient or physician delay. The asymptomatic patient was a seventy-year-old male with no cardiovascular risk factors besides uncontrolled hypertension and a BMI of 30.5 kg/m². He had no previous history of cardiovascular problems. Indication for CEA surgery was significant stenosis ($\geq 80\%$) of the carotid artery.

Table 1 | Patient characteristics

Patient characteristics and cardiovascular risk factors in culprit and non-culprit group, the asymptomatic patient not included.

CEA = carotid endarterectomy

a: defined as systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg.

Characteristic, n (%)	Culprit plaque 11 (77%)	Non-culprit plaque 3 (23%)
Sex, n (%)		
Female	3 (27%)	1 (33%)
Male	8 (73%)	2 (67%)
Age (years)		
Median (range)	73 (63-79)	67 (66-70)
Symptomatology, n (%)		
Cerebrovascular accident	4 (36%)	2 (67%)
Transient ischemic attack	5 (45%)	-
Amaurosis fugax	1 (9%)	1 (33%)
Time-interval between symptoms and CEA (weeks)		
Median (range)	2 (2-4)	7 (5-8)
Degree of stenosis (%)		
Median (range)	70% (50-99)	80% (70-99)
Dyslipidemia, n		
None	-	-
Controlled ≤ 2 drugs	6	3
Uncontrolled or controlled > 2 drugs	5	-
Diabetes, n		
None	9	2
Controlled with 1 drug	-	1
Uncontrolled or controlled > 1 drug	2	-
Hypertension ^a , n		
None	3	1
Controlled ≤ 2 drugs	3	1
Uncontrolled or controlled > 2 drugs	5	1
BMI (kg/m ²)		
Median (range)	26.2 (20.5-29.4)	26.9 (23.5-33.1)
Smoking status, n		
None or last smoked > 5 years	5	2
Current or last smoked ≤ 5 years	6	1
Cardiovascular history, n		
None	4	2
Second-degree family	-	-
First-degree family or own history	7	1

Fluorescent signal assessment

Prior to incubation, specimens were immediately imaged following a standardized imaging protocol to detect potential background signal. PEARL images of background signal in culprit plaques ($MFI_{background} = 1961$) was similar to non-culprit plaques ($MFI_{background} = 1883$). Specimens were then incubated within 20 minutes after surgical excision at room temperature with bevacizumab-800CW $1\mu\text{g}/\text{ml}$ for 1 hour. After incubation, the specimens were carefully rinsed, and subsequently imaged within 10 minutes. All PEARL images of culprit plaques showed clear fluorescent hot spots ($MFI = 36525$; range 22478 – 55530), whereas non-culprit plaques displayed less distinct, weak hot spots ($MFI = 8855$; range 3515 – 16586). MFI was calculated based on three to five measuring points per plaque depending on plaque size. After formalin-fixation and paraffin-embedding (FFPE) and micrometer tissue sectioning, flatbed scanning showed again a clear fluorescence pattern in culprit plaques with clear overlap of VEGF-A expression and the fluorescent signal pattern (Figure 1A shows a representative example). Based on the H&E staining, fluorescence microscopy revealed the presence of bevacizumab-800CW to be mainly intracellular (Figure 1B). The corresponding median MFI of the culprit plaques was higher (median $MFI = 42129$) than the median MFI of the non-culprit plaques (median $MFI = 6450$; Figure 2A), calculated for whole tissue fragments.

Figure 1 | Ex vivo fluorescent signal analyses of a culprit and non-culprit plaque

Odyssey (800nm) scans of a culprit (top row) and non-culprit plaque (bottom row) tissue sections, and the corresponding VEGF-A staining of the sections (VEGF-A is stained blue) (A). The sections were taken at approximately 1–2mm depth of the whole tissue specimens. Fluorescent intensity measurements are highest at sites of strong VEGF-A staining, as seen in the culprit plaque. On the contrary, the non-culprit plaque displays low fluorescent intensity and has no visible VEGF-A expression. Fluorescence microscopy of a culprit plaque demonstrates the near-infrared signal of the tracer (red; 800nm channel; left column) overlaps the cell cytoplasm, indicating the

intracellular localization of bevacizumab-800CW (H&E staining; right column). Cell nuclei are displayed by Hoechst staining (blue; DAPI channel; left column). The asterisk indicates the same place in the corresponding specimens (B).

VEGF-A immunohistochemistry

VEGF-A staining was performed in 30 sections of the total number of CEA specimens, with two sections per specimen. The calculated mean H-score of culprit plaques was 223 ± 25.1 ; the mean of the non-culprit group was 107 ± 6.34 . In 91% of the culprit plaques a strong staining intensity (H-score: 201-300) was found. The H-score of the plaque from the asymptomatic patient was 236, indicating an intense staining for VEGF-A. VEGF-A expression levels were observed to be clearly higher in the culprit plaques than in the non-culprit plaques (Figure 2B). In the culprit plaques, the pattern of fluorescence overlapped with VEGF-A expression in 82% of the specimens ($n = 9/11$) (Figure 2C).

Figure 2 | Fluorescent pattern intensity of tissue sections and VEGF-A staining intensity

Based on whole tissue fragments, a higher mean fluorescence intensity of all culprit plaques (36425; range 22478 – 55530) was observed in comparison to non-culprit plaques (8855; range 3515 – 16586) after incubation with bevacizumab-800CW. The median MFI of the culprit plaques was higher than the median MFI of the non-culprit plaques (42129 vs. 6450) (A). VEGF-A staining intensities (H-score) of both culprit and non-culprit plaques displayed by a boxplot. The average H-score was the highest in the culprit plaques (B). Representative example of a tissue section taken at approximately 1-2mm depth of a culprit plaque hot spot displaying the overlap of the pattern of fluorescence and VEGF-A immunohistochemistry (blue staining) (C).

Correlation parameters

Histological examination of the asymptomatic plaque revealed clear pathological features of vulnerability similar to culprit plaques as compared to non-culprit plaques such as intraplaque hemorrhage and intraplaque angiogenesis (Figure 3). In this particular plaque, intraplaque hemorrhage was present as shown by H&E staining (Figure 3B), a prominent feature of the vulnerable plaque.¹⁵ The asymptomatic plaque could therefore be deemed vulnerable, i.e. culprit, as these features are characteristic for culprit lesions.³² In Table 2, an overview of the various associations between VEGF-A staining and correlation parameters is displayed.

Table 2 | Correlation parameters

Correlation parameters for VEGF-A staining and CD34, CD68, SMA and Von Kossa staining

a: The Chi Square tests of independence were calculated based on the VEGF-A ROIs found in the specimens and the intensity of the various staining in those ROIs. The asymptomatic plaque was not taken into account.

b: For the association between SMA and VEGF-A staining, an inverse Chi Square test was used.

Characteristic	Culprit specimens, <i>n</i> (%)	Non-culprit specimens, <i>n</i> (%)	Chi Square tests of independence ^a
CD34 stain	15/15 (100%)	0/6	χ^2 (1) = 10.313 $p = 0.001$ $\phi = 0.829$ $n = 21$
CD68 stain	15/17 (88%)	1/4 (25%)	χ^2 (4) = 11.030 $p = 0.026$ $\phi = 0.606$ $n = 21$
SMA stain^b	2/17 (11%)	6/6 (100%)	χ^2 (4) = 11.341 $p = 0.023$ $\phi = 0.870$ $n = 23$
Von Kossa stain	16/22 (72%)	3/6 (50%)	χ^2 (1) = 1.116 $p = 0.291$ $\phi = 0.200$ $n = 28$

CD34 staining for microvessel density confirmed the presence of microvasculature in all culprit plaques. In addition, in all culprit plaques, including the asymptomatic plaque, VEGF-A staining was strongest in areas of high microvessel density. A strong association with VEGF-A staining was demonstrated ($\phi = 0.829$; $p < 0.001$). No intraplaque micro vessels were observed in the non-culprit plaques.

Von Kossa staining for (micro)calcifications was performed in 22 sections of the culprit plaques (2 per specimen), and revealed small calcium deposits around the VEGF-A and micro vessel areas in 73% of the sections ($n = 16/22$). Of the non-culprit plaques, 6 sections were stained with Von Kossa and 3 specimens revealed microcalcifications in both the intimal and medial layers. No significant association was found between microcalcifications and VEGF-A expression in both the culprit and non-culprit group ($p > 0.05$).

Macrophage infiltration (CD68 staining) was most abundant in the VEGF-A ROIs in culprit plaques ($n = 15/17$). Weak to moderate staining was found in non-culprit plaques. A moderate association was found between VEGF-A expression scores and CD68 staining scores ($\phi = 0.606$; $p < 0.05$). The average CD68 score in the culprit group was higher than in the non-culprit group (2.83 ± 0.389 vs 1.27 ± 0.577).

Strong expression of smooth muscle actin (SMA) staining was observed in the non-culprit plaques as opposed to mostly weak staining in culprit plaques. The asymptomatic plaque also displayed low SMA expression. There was a strong inverse correlation between H-scores and SMA staining ($\phi = 0.870$; $p = 0.023$).

Figure 3 | Representative examples of histologic parameters of hot spots of a culprit, the asymptomatic and a non-culprit plaque

The stainings were performed on subsequent slides, depicting a ROI in the specimens. H&E stain, demonstrating extensive intraplaque hemorrhage in the culprit plaque (the yellow asterisks indicate areas with red blood cells, orange spots) (A), (B) and (C); VEGF-A stain, revealing elevated VEGF-A expression quantified by the H-score in both the culprit and asymptomatic plaque as opposed to the non-culprit plaque (D), (E) and (F); CD34 stain indicating the association between VEGF-A and microvessel density (G), (H) and (I); CD68 stain, demonstrating an influx of macrophages around the micro vessels in the culprit and asymptomatic plaque (J), (K) and (L); Von Kossa stain, revealing no differences between the culprit, asymptomatic and non-culprit plaques (M), (N) and (O); SMA stain, showing elevated SMA expression in the non-culprit plaque (P), (Q) and (R).

DISCUSSION

Optical imaging is a relatively new modality in the clinical cardiovascular field and can be used for real-time detection of pathological processes.^{13,31,35} Currently, no decisive method is available to make a distinction between culprit and non-culprit atherosclerotic plaques. This study investigated the feasibility of using bevacizumab-800CW for the identification of vulnerable plaques by determination of their VEGF content. We demonstrated a successful method to detect a measurable fluorescent signal in incubated atherosclerotic plaques. Next, the optimal incubation concentration was determined, 1 µg/ml bevacizumab-800CW. We were able to demonstrate that areas of high VEGF-A expression, based on specific immunohistochemistry, correlated with a high fluorescence signal. Strong VEGF-A expression was observed in culprit plaques, in contrast to the weak staining found in non-culprit plaques.

We demonstrated the relationships between VEGF-A expression, and macrophage infiltration and intraplaque microvessel density. Although macrophage infiltration by itself does not appear to be predictive of upcoming acute events in the future, we found that it coincides with VEGF-A expression and intraplaque angiogenesis.¹⁵ No correlation was demonstrated between microcalcifications and VEGF-A expression. Furthermore, microcalcifications were present both in culprit and non-culprit plaques. This corresponds to previous research by Hop *et al.*, who investigated the use of the PET-tracer ¹⁸F-NaF specific for microcalcifications in culprit and non-culprit carotid plaques *ex vivo*.¹³

The inverse association found between VEGF-A expression and SMA expression demonstrates that SMA expression is more pronounced in non-culprit plaques, indicating these plaques to contain more mature vascular smooth muscle cells (VSMCs). This complements earlier research in which diminished SMA staining was related to unstable coronary plaques.³⁶ Additionally, Shinde *et al.* demonstrated that SMA-expressing myofibroblasts are associated with scar contraction in healing myocardial infarcts.³⁷ A possible explanation why SMA expression in particular is present in non-culprit plaques is that these myofibroblasts take part in wound healing processes and vessel repair, that presumably stabilize the atherosclerotic plaque.³⁸ Although VSMCs and myofibroblasts are distinct cell types, SMA may have protective properties in the sense that SMA expression stabilizes the atherosclerotic plaque.

The fluorescent signals found at sites of clear visual macrocalcification without VEGF-A expression at those specific sites could be explained by autofluorescence of calcified areas, as described previously in human atherosclerotic arteries. Conversely, the frail integrity of 3 µm tissue slides results in disrupted tissue sections due to tearing and breaking of the tissue. This could attribute for a weaker bevacizumab-800CW fluorescent signal, imaged by the flatbed scanner, at the corresponding sites of VEGF-A expression. Another reason could be the submersion method of incubation with bevacizumab-800CW as the tracer might not be able to penetrate the full thickness of the specimens.

A limitation of this study is the low number of asymptomatic and non-culprit atherosclerotic plaques, which posed a difficulty in comparing symptomatic to non-symptomatic plaques and assessing statistical significance between culprit and non-culprit plaques. Asymptomatic plaques are normally not resected as standard of patient care because of the patient risk involved in the procedure. The number-needed-to-treat (NNT) to prevent major CVA or death is 6 for symptomatic patients, whereas asymptomatic patients have an NNT of 20, emphasizing patient risk.^{39,40} Additionally, symptomatic plaques are normally removed within 2 weeks after symptom onset as standard of patient care in the Netherlands, which could be an explanation for the low number of non-culprit plaques.

This study complements previous research by our group with ⁸⁹Zr-bevacizumab and the use of bevacizumab in CEA specimens, as the monoclonal antibody bevacizumab was able to target VEGF-A *ex vivo* in human carotid atherosclerotic plaques in this study too.³¹ In addition, this study adds knowledge as we demonstrate the ability of the conjugate bevacizumab-IRDye800CW to identify intraplaque angiogenesis by targeting VEGF-A, and to be capable of distinguishing between culprit and non-culprit plaques. Furthermore, we demonstrate that angiogenesis correlates with macrophage infiltration using CD68 immunohistochemistry. An advantage is that a systemic dose bevacizumab-800CW is already proven to be safe and effective in humans, according to various cancer studies, which facilitates further *in vivo* research in the cardiovascular field.^{27–30} In general, the benefit of NIRF-tracers over PET-tracers is that the production process of NIRF-tracers is generally simpler and less expensive, due to the long shelf life and the elimination of radioactive infrastructure in the production process. NIRF-imaging allows for non-invasive imaging techniques, which is favorable in this fragile patient group. Additionally, with NIRF imaging, it is easier to image in real-time, there is no radiation burden, and the imaging time itself is relatively short, however whole body imaging and deep signal penetration is lacking.

In conclusion, the NIRF tracer bevacizumab-800CW is promising in visualizing VEGF-A expression in human carotid atherosclerotic plaques *ex vivo*. Our results demonstrate the potential of bevacizumab-800CW to define vulnerable (VEGF-positive) and non-vulnerable (VEGF-negative) plaques. The validation of the latter hypothesis should take place in a clinical study. Future studies should focus on the clinical proof of principle *in vivo* of the use of bevacizumab-800CW in this particular patient group, to allow for its validation *in vivo*. We believe that this tracer might allow for improved risk-stratification of carotid atherosclerosis and therefore a reliable selection method for CEA. Benefits would be prevention of disease and mortality caused by acute vascular events. Ultimately, clinical implementation of this tracer to select for CEA should rely on the predictive value of angiogenesis in high-risk plaques.

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CHAPTER 7

VEGF-targeted multispectral optoacoustic tomography and fluorescence molecular imaging in human carotid atherosclerotic plaques

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ABSTRACT

Vulnerable atherosclerotic carotid plaques are prone to rupture, resulting in ischemic strokes. In contrast to radiological imaging techniques, molecular imaging techniques have the potential to assess plaque vulnerability by visualizing diseases-specific biomarkers. A risk factor for rupture is intra-plaque neovascularization, which is characterized by overexpression of vascular endothelial growth factor-A (VEGF-A). Here, we study if administration of bevacizumab-800CW, a near-infrared tracer targeting VEGF-A, is safe and if molecular assessment of atherosclerotic carotid plaques *in vivo* is possible using multispectral optoacoustic tomography (MSOT). Healthy volunteers and patients with symptomatic carotid artery stenosis scheduled for carotid artery endarterectomy were imaged with MSOT. Secondly, patients were imaged two days after intravenous administration of 4.5 mg bevacizumab-800CW. *Ex vivo* fluorescence molecular imaging of the surgically removed plaque specimen was performed and correlated with histopathology. In this first-in-human MSOT and fluorescence molecular imaging study, we show that administration of 4.5 mg bevacizumab-800CW appeared to be safe in five patients and accumulated in the carotid atherosclerotic plaque. Although we could visualize the carotid bifurcation area in all subjects using MSOT, bevacizumab-800CW-resolved signal could not be detected with MSOT in the patients. Future studies should evaluate tracer safety, higher doses of bevacizumab-800CW or develop dedicated contrast agents for carotid atherosclerotic plaque assessment using MSOT.

INTRODUCTION

Carotid atherosclerotic plaque rupture is a major cause of ischemic stroke, accounting for 18-25% of all stroke events.¹ Currently, carotid endarterectomy (CEA) is recommended if the degree of extracranial internal carotid artery (ICA) stenosis is >70% in symptomatic patients to prevent a second ischemic stroke event or death.² However, some ischemic strokes do not correlate with stenosis severity but with plaque rupture.³

Identification of these 'vulnerable plaques' by obtaining information on plaque composition could be a valuable tool to select symptomatic and eventually asymptomatic patients with increased risk for ischemic stroke that would benefit from a CEA.⁴ There is strong evidence that vulnerable plaques show increased levels of inflammation, characterized by a thin fibrous cap, a necrotic core and increased macrophage infiltration.^{5,6} Particularly, vulnerable plaques show intra-plaque neovascularization, induced by the intra-plaque release of vascular endothelial growth factor-A (VEGF-A).^{6,7} Earlier, our group showed uptake of the nuclear tracer ⁸⁹Zr-bevacizumab and the near-infrared fluorescent tracer bevacizumab-800CW, both targeting VEGF-A that linked to plaque vulnerability. Successful imaging of VEGF-A to identify vulnerable, unstable plaques has been shown through Positron Emission Tomography.^{7,8}

Optoacoustic imaging is a novel non-invasive imaging modality that detects absorption-induced pressure or optoacoustic waves. As such, optoacoustic imaging enables molecular imaging at depths of several centimeters with high spatial resolution.⁹ Specifically, multispectral optoacoustic tomography (MSOT) can detect multiple photoabsorbers in tissue, including both intrinsic tissue chromophores (e.g. hemoglobin, deoxyhemoglobin) and exogenous contrast agents. In contrast to ultrasound (US), optoacoustic imaging has the potential to provide biological information on plaque composition, which may improve patient stratification for CEA. To date, the feasibility of MSOT for vascular imaging, including intravascular imaging, has been shown in multiple studies.¹⁰⁻¹⁴ Ivankovic *et al.* showed visualization of the carotid artery with high sensitivity and spatial resolution *in vivo* using optoacoustic imaging.¹⁰ More recently, MSOT was used for carotid plaque visualization.¹⁵ Here, we study if an intravenous injection of bevacizumab-800CW, a near-infrared fluorescent tracer that has been used in pilot studies in surgical oncology, is safe in patients undergoing CEA, a vulnerable patient population with multiple risk factors of cardiovascular disease.^{16,17} We aim to determine if *in vivo* MSOT of bevacizumab-800CW is feasible in symptomatic patients with carotid atherosclerotic plaques.

MATERIALS AND METHODS

Clinical trial design

This microdosing safety and proof-of-concept study was performed at the University Medical Center Groningen. Healthy volunteers were recruited and underwent MSOT without prior tracer administration to study technical feasibility. Subsequently, symptomatic patients (i.e. after a cerebrovascular accident or a transient ischemic attack) scheduled for CEA were included. Patients received an intravenous microdose of 4.5 mg bevacizumab-800CW three days before surgery. In this vulnerable patient cohort, an FDA-approved microdosing strategy with no direct

pharmaceutical effect which gives insight in the pharmacokinetics was used to grant the safest environment for patients while still being able to study the binding of the tracer.¹⁸ The used time interval (3 days prior to surgery) is used in a variety of oncological studies using bevacizumab-800CW based on the optimal imaging period due to the antibody half life. MSOT was performed before and two days after tracer administration. Two carotid artery specimens from consenting symptomatic patients without tracer administration were used as a negative control. The primary endpoints were tracer safety and feasibility of bevacizumab-800CW visualization with *in vivo* MSOT and *ex vivo* fluorescence molecular imaging. The study workflow is summarized in Figure 1.

Approval for this study was obtained at the Institutional Review Board (IRB) of the UMCG (METc 2018/477). The study was performed in accordance with the Helsinki Declaration (adapted version 2013, Fortaleza, Brazil). Written informed consent was obtained from all individual participants included in the study. The trial was registered at the Clinical Trials Register (NCT03757507).

Figure 1 | Imaging workflow

MSOT was performed on healthy volunteers for technical feasibility (A). On day 0, optoacoustic imaging and tracer administration were performed, followed by bevacizumab-800CW-targeted optoacoustic imaging one day prior to carotid endarterectomy (B). Afterward, tissue processing using fluorescence imaging of the carotid specimen was performed (C). Abbreviations: MSOT, multispectral optoacoustic tomography; CCA, common carotid artery; ECA, external carotid artery; ICA, internal carotid artery.

Safety data

Safety for this vulnerable patient group was a primary endpoint of this clinical study, both regarding MSOT and bevacizumab-800CW administration. Bevacizumab-800CW was in-house produced in the Good Manufacturing Practice facility of the UMCG, as previously described in detail.¹⁹ Vital signs and physical examinations were obtained before and after MSOT and bevacizumab-800CW administration. Two weeks after tracer administration, patients were contacted by phone to identify adverse events, according to the National Cancer Institute CTCAE version 5.0.²⁰

Optical characterization of bevacizumab-800CW

Prior to the start of the clinical study, the optoacoustic profile of bevacizumab-800CW for *in vivo* use was studied. A tissue-mimicking phantom was fabricated using using 300 ml deionized water, 2% agarose and 6% intralipid to mimic the optical properties of biological tissue. Polyethylene tubes with a diameter of 3 mm containing bevacizumab-800CW samples (diluted in deionized water) with a decreasing concentration ranging from 6.67 μM to 13 nM were inserted at a depth of ~ 1 cm. An IRDye800W sample with an optical density of 2 was additionally imaged as a reference. The phantom was placed in a water bath to ensure adequate coupling between the transducer and the phantom, and the samples were imaged with wavelengths from 660 to 900 nm in steps of 10 nm. Circular regions of interest (ROI)s of the same size were placed over the samples in the MSOT images, and subsequently duplicated at the same depth in the background to serve as a reference. The optoacoustic spectrum of bevacizumab-800CW was determined based on the highest concentration sample and compared with the IRDye800CW optoacoustic spectrum. Secondly, the maximum optoacoustic signal measured at 780 nm (i.e. optoacoustic peak) was plotted against molar concentration with background signal as a reference.

Optoacoustic imaging

All MSOT procedures were performed using a clinical research hybrid ultrasound (US) - MSOT system (MSOT Acuity Echo prototype; iThera Medical GmbH, Germany). This system uses a 25 Hz pulsed Nd: YAG laser for emission of 25 mJ pulses, which has shown clinical safety in multiple studies.²¹ The two-dimensional (2D) concave detector (4-MHz center frequency, 256 transducer elements) provides cross-sectional imaging with an in-plane spatial resolution of ~ 180 μm and field of view of 40x40 mm. Simultaneously, US signal is detected for anatomical guidance of the imaging procedure.

Imaging of the common carotid artery (CCA), the carotid bifurcation and the internal and external carotid artery was performed. Subjects were in a supine position with the neck in hyperextension and the chin rotated to the contralateral side. MSOT signals were acquired using a preset with six wavelengths (700nm, 730nm, 760nm, 780nm, 800nm and 850nm) to sample the absorption spectrum profiles of hemoglobin (HbO₂) deoxyhemoglobin (HbR) and IRDye800CW.

MSOT data were analyzed using cLabs (iThera Medical GmbH) and reconstructed using a back-projection algorithm. The lumen of the CCA and the plaque in patient's data were manually segmented in the US image. The lumen and plaque ROIs were duplicated at the same depth in the background region to serve as a reference. Optoacoustic signals in arbitrary units (a.u.) were extracted from the ROIs on three consecutive frames and average optoacoustic signals were registered. For image reconstruction, single wavelength analyses were performed at 800nm to detect both HbO₂ and HbR, 850nm to detect HbO₂ and 780 nm to detect IRDye800CW.²² In addition, spectral unmixing was performed to study HbO₂, HbR and IRDye800CW signal.

For quantitative analysis, the mean intensity ratios between the ROI and the background ROI were calculated to allow for data comparison of different patients, as reported earlier.¹⁵ As such, the effects of imaging depth and light fluence are minimized.

Ex vivo analyses of surgical specimen

Directly after CEA, the atherosclerotic plaque was imaged using a closed-field fluorescence camera system (PEARL Trilogy, LI-COR Biosciences, Lincoln, NE, USA). Subsequently, the specimen was formalin-fixed for 24 hours and serially sliced in ~1 mm thick tissue slices. Fluorescence flatbed scanning of tissue slices was performed using the Odyssey® CLx (LI-COR Biosciences) and embedded in paraffin blocks (formalin-fixed paraffin-embedded [FFPE]-blocks).

ROIs were drawn of the plaque within the surgical specimen using white light images with plaque anatomy as a reference. Mean fluorescence intensities (MFI) of the bevacizumab-800CW group and the negative control from all tissue slices (2-3 tissue slices per patient) were calculated. 4 µm tissue sections were obtained of all FFPE-blocks and hematoxylin and eosin (H&E) staining and VEGF immunohistochemistry were performed to correlate imaging results with histology. A pathologist (GFHD) blinded for imaging results analyzed all H&E tissue sections.

To assess the presence of intact bevacizumab-800CW in the atherosclerotic plaque, we performed sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-page) on one sample of a fresh frozen surgical carotid artery specimen, as previously described. Bevacizumab-800CW was yielded in concentrations of 10 to 80 µg total protein on a biorad mini protean TGX precast gel (7.5%). Gels were stained with imperial protein stain (Thermo Scientific). Unlabeled bevacizumab was used as a reference. Subsequently, we scanned the gel using the 800 nm channel of the Odyssey CLX® flatbed scanning system.

Statistics

Due to this proof-of-concept study's explorative character and the subsequent small sample size, only descriptive statistics were used. MFI was defined as total counts per ROI pixel area (signal/pixel). Descriptive statistics and graph design were conducted using Graphpad Prism version 8.

RESULTS

Validation of bevacizumab-800CW detection using a clinical MSOT system

The optoacoustic spectrum of bevacizumab-800CW and IRDye800CW were determined by imaging the different samples in a tissue-mimicking phantom at ~1 cm depth. Optoacoustic spectra were normalized to a maximum of 1. Both samples showed optoacoustic peaks at ~700 and ~780 nm, with bevacizumab-800CW showing slightly higher signal at ~700 nm, similar to previous studies with IRDye800CW conjugated to a monoclonal antibody (Figure 2). The second of IRDye800CW-peak at ~700 nm has previously been identified, and is possibly caused by stronger optoacoustic generation efficiency attributable to H-aggregates.²³

A strong correlation was found between maximum optoacoustic signal observed with MSOT and bevacizumab-800CW concentration (Pearson $r = 0.96$). Bevacizumab-800CW could be differentiated from the background signal down to a limit of 416 nM.

Figure 2 | Optoacoustic characterization of bevacizumab-800CW

A) Optoacoustic spectra of bevacizumab-800CW and IRDye800CW in a tissue-mimicking phantom at ~1 cm depth as determined with the MSOT Acuity Echo prototype. Two optoacoustic peaks are detected at 700 and 780 nm, with bevacizumab-800CW showing higher signal at 700 nm. B) Ultrasound and MSOT 780 nm images of one of the samples imaged with corresponding ROIs of samples (blue) and background (red). C) MSOT spectra of bevacizumab-800CW and IRDye800CW show two distinct peaks at 700 and 780 nm, with bevacizumab-800CW showing higher signal at 700nm. D) Maximum optoacoustic signal observed with MSOT showed strong correlation with bevacizumab-800CW concentration (Pearson $r = 0.96$). The detection limit for bevacizumab-800CW in this setup was 416 nM.

Subject characteristics and safety data

Five healthy volunteers and five patients with symptomatic carotid artery stenosis were included in this study. Since we could not detect bevacizumab-800CW with MSOT *in vivo*, the trial was stopped before completing the target patient sample size (n=10). In total, four patients (all males, mean age 76) underwent the complete *in-* and *ex vivo* imaging protocol. For one patient who received bevacizumab-800CW, the surgical plan was converted to carotid artery stenting resulting in no surgical specimen obtainment. In all patients, the plaque was localized in the internal carotid artery and CCA in at least partially involved the ventral wall of the CCA. Patient and healthy volunteer characteristics and safety data are shown in Table 1. No drug-related or imaging related (serious) adverse events were observed .

Table 1 | Clinical characteristics of healthy volunteers and patients who underwent bevacizumab-800CW administration and finished the study

Data are presented in numbers with percentages (%) or means with range.

Characteristics	5 volunteers
Age (years), mean (range)	28 (23-38)
Sex: males, n (%)	5 (100)
Weight (kg), mean (range)	82 (74-86)
Height (cm), mean (range)	186 (172-189)
Imaging time (min) mean (range)	11 (5-12)
MSOT related adverse events	0

Characteristics	4 patients
Age (years), mean (range)	76 (58-82)
Sex: males, n (%)	4 (100)
Weight (kg), mean (range)	71.5 (65-80)
Height (cm), mean (range)	172.5 (170-178)
Total infusion time (min)	1
Imaging time (min), mean (range)	9 (5-12)
Adverse events (grade 1-4)	0
MSOT related adverse events	0
Total tissue slices analysed	43

***In vivo* MSOT**

Healthy volunteer imaging showed the technical feasibility of *in vivo* optoacoustic visualization of the carotid artery and detection of intrinsic tissue chromophores such as HbR and HbO₂ (Supplemental Figure 1). In all patients, handheld MSOT enabled real-time visualization of the CCA, internal carotid artery and external carotid artery, and identification of the atherosclerotic plaque. Although the plaque was localized in the internal carotid artery and CCA in all patients, we used only the CCA for analyses, as the light fluence in the internal carotid artery was limited by absorption of the external carotid artery. Strong absorption of hemoglobin restricted further penetration light, resulting in a “cap” of optoacoustic signal in the CCA. In all images, the optoacoustic intensity in the CCA was higher than that of surrounding tissue, both when using single wavelength analyses and spectral unmixing. (Figure 3A).

Subsequently, we assessed the ability of MSOT to identify vulnerable plaques by detecting bevacizumab-800CW signal within the atherosclerotic plaque. Pre-injection and post-injection data of single wavelength analysis at 780 nm and IRDye800CW spectral unmixing were analyzed per patient. In one patient, no pre-injection MSOT was performed due to the unavailability of the dedicated MSOT room, resulting in a total of seven data points. Upon spectral unmixing, no optoacoustic signal of bevacizumab-800CW could be detected with MSOT. Also, no increase in single wavelength analysis of 780 nm was observed after tracer administration (1.13 (IQR 1.00-1.27) a.u. post-injection vs 1.26 (IQR 0.79-1.17) a.u. pre-injection).

Figure 3 | Macroscopic optoacoustic and fluorescence imaging

Representative hybrid optoacoustic image of carotid atherosclerosis pre- and postinjection (A), the region of interest resembling the carotid plaque used for measurements is delineated with a red line. Pre- and postinjection measurements of 780 nm optoacoustic imaging of all carotid arteries showed no difference on 780 nm and IRDye800CW. Fluorescence imaging of a whole surgical carotid specimen and a formalin-fixed tissue slice with standard histopathology (B). Mean fluorescence intensities (PEARL) of the plaque in all surgical tissue slices from negative patients and bevacizumab-800CW patients. Abbreviations: MSOT, multispectral optoacoustic tomography; HbR, deoxygenated hemoglobin; HbO₂, oxygenated hemoglobin; HbT, total hemoglobin.

Ex vivo analyses

To further evaluate specific bevacizumab-800CW tracer uptake in the atherosclerotic plaque, we performed closed-field fluorescence imaging of the freshly excised surgical specimen. Macroscopically, fluorescence signal correlated well with areas within the plaque containing calcifications (Figure 4A). To evaluate whether the fluorescence signal was derived from the tracer, we included two carotid plaque specimen of consenting negative controls. Tissue slices of the experimental group showed an MFI of 2.37 (IQR 1.90 - 3.99) $\times 10^{-4}$ compared to 1.86 (0.79 - 2.26) $\times 10^{-4}$ in negative controls (Figure 3B).

Analysis of $10\text{ }\mu\text{m}$ sections showed increased MFI compared to the vascular wall (Figure 4A). In the atherosclerotic plaques, mainly large fibrotic and necrotic tissue was observed upon H&E histopathological examination (Figure 4B). A representative image of high VEGF-A expression in the carotid artery is demonstrated in correlation with a fluorescence signal (Figure 4B). The presence and integrity of bevacizumab-800CW within the excised vulnerable plaque was demonstrated using SDS-page on a fresh frozen excised specimen (Supplemental Figure 2).

Figure 4 | Microscopic tracer distribution

Fluorescence imaging of a formalin-fixed paraffin-embedded carotid tissue section of a vulnerable atherosclerotic plaque (A, B). Both regions with VEGF-A overexpression and calcifications due to autofluorescence show high fluorescence signal, which cannot be attributed to VEGF-specific signal (B). Abbreviations: VEGF, vascular endothelial growth factor A.

DISCUSSION

Currently, no imaging method can differentiate vulnerable from non-vulnerable plaques with adequate accuracy for clinical use to assess for risk stratification and patient selection prior to surgery. To our knowledge, this is the first proof-of-concept study that aimed to visualize vulnerable atherosclerotic plaques by MSOT using bevacizumab-800CW for VEGF-A expression as a biomarker of intra-plaque neovascularization. Our data demonstrate that microdosing 4.5 mg bevacizumab-800CW is safe in a vulnerable patient population undergoing CEA. Although we could not detect bevacizumab-800CW-specific signal through *in vivo* MSOT, accumulation of intact bevacizumab-800CW in the carotid plaque specimen was objectified with SDS-page.

In all patients, we could visualize the entire carotid artery bifurcation area and identify the atherosclerotic plaques. Using both single wavelength analyses and spectral unmixing we could differentiate several intrinsic tissue chromophores, regardless of different imaging depths affecting light fluence. However, 4.5 mg bevacizumab-800CW could not be detected with MSOT to visualize intra-plaque neovascularization within plaques. Further *ex vivo* analysis with fluorescence imaging systems neither proved bevacizumab-800CW-specific signal. Nevertheless, the accumulation of bevacizumab-800CW was demonstrated through SDS-page (Supplemental Figure 2). We surmise that a microdose bevacizumab-800CW does not result in a detectable increase in optoacoustic signal, or fluorescence signal surpassing plaque autofluorescence.

The findings of this study are in line with previous studies that showed accumulation of bevacizumab in carotid atherosclerotic plaques expressing VEGF-A. Recently, our group has shown that the conjugate bevacizumab-800CW can discriminate between vulnerable and non-vulnerable plaques, and identify intraplaque angiogenesis by targeting VEGF-A.²⁴ Although the accumulation in atherosclerotic plaques was detected with ⁸⁹Zr-bevacizumab micro-PET in *ex vivo* CEA specimens, which in fact correlated with VEGF immunohistochemistry scores, we could not detect bevacizumab-800CW in the current dose with neither MSOT or fluorescence imaging.^{8,16,25}

We hypothesize that the inability to detect bevacizumab-800CW with MSOT or fluorescence imaging is due to two causes. First, the current microdose might be too low to be detected or surpass the autofluorescence in the atherosclerotic plaque. Secondly, the photophysical properties of the tracer are not optimal for optoacoustic signal generation. Therefore, we propose two strategies for future clinical studies that pursue MSOT for identification of vulnerable plaques by targeting VEGF-A, in which is used as targeting moiety since it has shown specific discrimination between vulnerable and non-vulnerable plaques.

The first and most straightforward approach could be a dose-escalation study employing an increasing dose of bevacizumab-800CW to approximate the detection limit of optoacoustic imaging, as has been carried out in multiple clinical trials in fluorescence-guided surgery and fluorescence-guided endoscopy. An alternative approach is to conjugate bevacizumab to a dedicated optoacoustic signaling compound, currently not clinically available but has gained increasing attention last decade.^{26,27} Optoacoustic contrast agents have been reviewed extensively and ideally exhibit a high molar extinction coefficient in the NIR, a characteristic absorption spectrum with sharp peaks, high photostability, high photothermal conversion efficiency, low quantum yield and favourable biocompatibility.²⁶

Our data processing framework is a step-up towards the standardization of dual modality optoacoustic imaging together with fluorescence imaging for visualizing plaque biology and atherosclerosis. Further studies for optimizing optoacoustic contrast agents are needed to enable non-invasive transcutaneous visualization of vulnerable plaques *in vivo*.

SUPPLEMENTAL DATA

Supplemental Figure 1 | Healthy Volunteer Imaging
Representative image of healthy volunteer carotid artery imaging

Supplemental Figure 2 | Gel electrophoresis
Representative image of intact bevacizumab-800CW at 150 kD. SDS-PAGE was performed on a fresh frozen tissue slice of a carotid surgical specimen collected directly after carotid endarterectomy.

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CHAPTER 8

Summary

General discussion

Future perspectives

SUMMARY AND GENERAL DISCUSSION

Cardiovascular diseases and cancer are both dominant causes of morbidity and mortality worldwide. In both entities, the development of medical imaging modalities like ultrasonography, CT, MRI and nuclear imaging modalities like PET/SPECT, has led to an improved pre- and intraoperative visualization of disease anatomy and biology. This led to an impressive increase in diagnostic, therapeutic and eventually surgical success during surgical staging, treatment and follow-up. The introduction of molecular imaging (MI), an innovative form of imaging modality, has shown the advantages of collecting precise images of disease behaviour on a molecular and cellular level. Targeting disease-specific biological characteristics using MI led to the discovery of novel tumor targets, novel oncological drugs and a disease-based and personalized treatment plan.¹⁻⁴ The discovery and validation of the efficacy of novel biologicals and immunotherapeutic drugs could potentially increase disease-free and overall survival rates in a majority of solid tumors.⁵ Surgery is, next to radio- and chemo/immunotherapy, one of the main pillars during oncological treatment, in which MI is able to visualize complex tumor biology prior and during surgery.⁶

The first part of this thesis focusses on surgical oncology. The main goal of an oncological surgical procedure is complete resection of the primary tumor including all surrounding microscopic tumor cells, the so-called tumor negative margin. However, despite technical advances, surgical margins often contain tumor cells (tumor-positive margins) in a variety of tumor types.⁷ These positive margins results lead to a decreased disease- and overall survival and mostly necessitate additional treatment (surgery and/or chemo/radiotherapy). MI has the potential to prevent irradicality in the future and offers a variety of opportunities to distinguish malignant from benign tissue. Tumor-targeted nuclear imaging has already shown that the integration of biomolecular information using radionuclides can improve cancer diagnostics and therapy by providing molecular tumor characteristics and labeled drug (radioactive or fluorescent) target interaction after treatment.⁸ Despite major progression, the successful implementation of tumor targeted imaging modalities (like CT, MRI and nuclear imaging) in the oncological surgical setting - the surgical theatre - to improve surgical outcome has not been established yet.

Intraoperative fluorescence imaging (FI) is a novel technique, which can detect (auto) fluorescence with dedicated imaging systems which can be incorporated during open, laparoscopic and robotic surgery. Today, surgeons (still) rely on their visual and tactile feedback during surgery, and lack tactile feedback during robotic and laparoscopic surgery. The additional value of anatomical and biological information provided by FI is obvious due to the inherent properties of optical imaging. Disease-targeted optical imaging agents, which accumulate in the tissue of interest (e.g. tumor tissue, metastatic lymph nodes) are promising for tissue characterization with high resolution. As imaging is performed on a relative small surface, FI incorporates real-time qualitative and quantitative imaging up to a few millimeters with high spatial and temporal resolution.^{9,10} When compared to more expensive and large nuclear imaging devices, FI fits easier into the standard workflow of the surgical theatre. Moreover, FI comes with lower costs and lacks radioactivity, which is normally onerous for the patient and the surgical staff. Tumor-specific FI has gained significant scientific and clinical interest in the past decade by

providing a real-time method to increase surgical quality after the first initial in-human clinical trial in 2011.^{11–13} Most trials have focused on targeting tumor-specific upregulated proteins using tumor biology in oncogenesis to detect tumor tissue and metastatic lymph nodes. The final goal is to perform adequate surgical margin assessment and setting new standards for tumor visualization and data analysis in favor of patients with solid tumors.¹⁴

FI, for example with the non-specific FDA-approved imaging agent indocyanine green (ICG), is widely used for the evaluation of perfusion during anastomotic surgery and to enhance tumor and sentinel node detection.^{15,16} However, there is no general consensus on the ICG dosing strategy, timing of imaging, qualification and quantification and no standardized imaging protocol defining ICG perfusion is available. Therefore, decisions only rely on subjective measurements of the presence of fluorescence and normalized signal intensities interpreted by not optimally trained surgeons, which increases significant observer bias. The aspecificity of ICG and the lack of standardization is a major drawback for standard of care use in surgical oncology. In this thesis, we use conjugated ICG in a tumor-targeted fashion in **chapter 3** and **4** and describe the first steps in standardization for tumor-specific decision making.

Improved and more reliable visualization techniques using MI are not only interesting in the field of oncology, but are also desirable in cardiovascular diseases, the focus of the second part of this thesis. Cardiovascular disease is the leading cause of death worldwide, taking an estimated 17.9 million lives each year.¹⁷ Duplex/doppler ultrasound is often the first non-invasive step in the vascular workup, with a high spatial and temporal resolution giving a high imaging sensitivity. However it is unable to visualize and quantify detailed biological information. Computed Tomography-Angiography (CT-A) is able to precisely visualize atherosclerosis. Magnetic Resonance-Angiography (MR-A) using a less toxic contrast agent (e.g. gadolinium) has a lower resolution and both are susceptible to artefacts (motion, implantable devices, vascular calcifications), limiting their interpretation.¹⁸ Nuclear imaging enables whole body imaging which is highly sensitive with a low tracer concentration, but also has a poor spatial resolution. The biological understanding of angiogenesis and vascular remodelling facilitates the potential to develop non-radiative clinical modalities to increase early diagnosis, which could improve patient selection prior to surgical or therapeutic interventions.¹⁹

Implementation of FI in vascular surgery is desirable but challenging, due to the limited penetration depth (max. 1 cm). Optoacoustic imaging, an innovative non-invasive modality, overcomes this challenge as it combines ultrasonography with optical illumination using pulsed laser light. The laser light is illuminated to the tissue of interest, where molecules absorb this energy and convert it into acoustic waves on a specific wavelength which can be detected using special transducers. Using this technique, both endogenous and exogenous contrast agents can be detected up to 5 centimeter in depth. Several clinical studies have described the advantages of optoatouscic imaging in oncology, inflammatory and vascular diseases.^{20,21} However, the introduction of optoacoustic imaging in clinical MI has not yet been established.

The aim of this thesis was to gain insight in the safety and the *in-* and *ex vivo* feasibility of novel fluorescence imaging agents in a standardized workflow in a variety of solid tumors (soft tissue sarcomas, head and neck cancer, breast cancer, colorectal cancer and esophageal cancer). We are aiming for a future in which surgical decision making can be improved directly during surgery using tumor-specific FI. Secondly, we evaluate the possibility of visualizing the so-called vulnerable atherosclerotic plaque using the fluorescent labeled antibody bevacizumab-800CW with *in vivo* optoacoustic imaging.

Chapter 1 provides a general introduction of fluorescence and optoacoustic imaging and the outline of this thesis. The first part of this thesis describes advances in intra-operative FI with the final goal to improve tumor-targeted imaging to reduce the high amount of tumor-positive surgical margins.

Targeting the overexpression of the protein vascular endothelial growth factor-A (VEGF-A) using bevacizumab-800CW in soft tissue sarcomas (STS) is described in **chapter 2**.^{22,23} We show the feasibility of *in-* and *ex vivo* visualization of STS using bevacizumab-800CW. All STS, but especially high-grade myxofibrosarcomas, are feared for their high rate of tumor-positive surgical margins (10-35%), causing increased local recurrence with major morbidity and a decrease in quality of life.²⁴ Although the clinical activities within the field of fluorescence-guided surgery (FGS) is increasing rapidly, this chapter describes one of the first tumor-targeted FGS studies in STS, whereas only tumor-aspecific ICG and the affibody ABY-029 targeting endothelial growth factor receptor (EGFR) have been investigated in STS.^{25,26} They showed accurate visualization of STS but did not provide tumor-specific quantification. Moreover, based on immunohistochemistry, EGFR seems not the most optimal or adequate target for STS, because STS show a high expression of tumor endothelial marker 1 (TEM1) and VEGF-A and a weak EGFR expression.²³ We observed that bevacizumab-800CW (10 mg flat-dose bolus administration) revealed intra-operative visualization with an *in vivo* tumor-to-background ratio (TBR) of 2.0 and an *ex vivo* TBR of 2.67. No tracer related adverse events were observed in all STS dosing groups (10, 25 and 50 mg flat-dose). We conclude that STS resection margin analysis immediately after excision has the potential to guide the surgeon to the closest adequate surgical margin, identifying the potential locations for immediate re-resection. In this study, all 7 tumor-positive margins could be detected using *ex vivo* imaging (so-called backtable imaging), emphasizing the positive effect of quick *ex vivo* imaging performed directly after excision. Necrotic tissue is provoked by neoadjuvant radiotherapy (RT), which also gives an increased risk of wound complications.²⁷ In this study, we observe fluorescent activation in necrotic tissue in our patients who underwent neoadjuvant RT. We therefore emphasize the need for FI during primary resection with radiotherapy in an adjuvant setting, potentially changing the standard treatment sequence in STS. These results will be used for a subsequent phase II study with the final goal to increase immediate tumor-positive margin detection and decrease morbidity and re-excisions after STS surgery.

In **chapter 3**, we describe the safety and feasibility for generic tumor imaging using a pH-activated nanoparticle-based imaging agent called ONM-100 in a first-in-human study. The tumor-targeting approaches with monoclonal antibodies like bevacizumab-800CW and cetuximab-800CW (target EGFR) are promising, but lack broad applicability as there is a large diversity in oncogenotypes and phenotypes between the various tumor types and even within one tumor type.²⁸ Moreover, due to the relatively long half-life time of monoclonal antibodies (i.e. 5-7 days), intravenous injection a few days prior to surgery is necessary (i.e. 2-3 days), which makes it less practical for clinical implementation. A method to overcome the challenge of tumor heterogeneity is to target a common denominator, namely low extracellular tumor pH, observed in all solid tumor types due to anaerobic glycolysis and tumor hypoxia.²⁹ This effect has already been observed in 1924 by dr. Warburg and is now described as one of the hallmarks of cancers.³⁰ ONM-100, a pH-activatable fluorescent imaging agent, consists of pH-sensitive polymers, where ICG is sequestered at the hydrophobic segments. The micelles dissociate in the acidic extracellular tumor microenvironment, creating fluorescent labeling of tumors over time.³¹ ONM-100 was considered safe in all five dosing groups (0.1 – 1.2) and showed a dose dependent increase in pharmacokinetics and contrast in 30 patients with several tumor types. A very sharp delineated tumor-specific fluorescence visualization was observed in all four tumor types investigated (breast cancer, head- and neck squamous cell cancer, colorectal cancer and esophageal cancer). The potential of ONM-100 for the complete removal of tumor tissue was highlighted with the detection of all tumor-positive margins (9/9, 100% sensitivity) during real-time *ex vivo* whole specimen and surgical cavity imaging. Moreover, four occult lesions, missed during initial surgery, were detected based on fluorescence imaging of the surgical cavity. In the current ongoing phase II study using ONM-100 (www.clinicaltrials.gov: NCT03735680), the timing of administration shortly prior to surgery and the implementation of additional types of solid tumors like ovarian cancer and prostate cancer is further investigated. As ONM-100 targets acidosis (a more generic tumor characteristic compared to tumor-specific overexpression of proteins), this pH-activatable nanoprobe may be used in a variety of tumors allowing broader clinical implementation of FI in the future as a ‘one-size-fits-all solution’.

By utilize fluorescent tracers, FI enables the surgeon to inspect the tumor directly after surgical excision. A standardized setting could help in adequate sampling of tumor tissue during pathology processing, potentially decreasing sampling error and applying immediate *ex vivo* tissue imaging, appointed by our group as intraoperative pathology assisted surgery (IPAS). We have observed a high amount of tumor-positive surgical margins in both mentioned clinical studies, possibly due to better sampling using fluorescence, as fluorescence imaging enables the pathologist with a tool to adequately select areas of interest for more precise (histo-) pathological sampling. This technique allows interpretation of data within minutes after surgical excision, potentially immediately influencing surgical strategy, as we will show in **chapter 4**. When a fluorescent-positive lesion indicative for a positive margin is observed, a decision for immediate re-excision can be made. The main advantage of IPAS is that imaging is performed in a standardized setting, which overcomes the *in vivo* imaging drawbacks as they are sterilization, camera to tissue distance, tissue characteristics and surrounding ambient light.³² One should

strive for immediate surgical decision making using easy-to-execute and easy-to-interpret imaging protocols. Surgeons need the availability of fluorescence cameras at the surgical theatre to improve decision making during oncological surgery and to prove that our reported phase 1 feasibility trials truly have clinical benefit.

The detection of a tumor-positive margin using fluorescence remains mostly subjective as no standardized imaging protocols are, yet, in place. In **chapter 4**, we describe the use of IPAS in patients with head- and neck cancer using ONM-100. The binary off/on mechanism due to nanoscale macromolecular cooperativity with a Hills coefficient of 51 generated a sharp delineated signal which could easily differentiate tumor tissue from non-tumor tissue. A cut-off TBR of >1.5 was used to evaluate whether a surgical margin was positive for fluorescence and using this technique we were able to localize all tumor-positive margins in an intra-operative setting, as confirmed by final histopathology. We suggest the use of this imaging analysis with a standardized *ex vivo* imaging system in combination with a tumor agnostic imaging agent for intraoperative margin evaluation.

Chapter 5 describes a novel potential of FGS to improve tumor detection and eventually patient selection for either limited or more extensive surgery in patients with peritoneal metastasis (PM) from colorectal origin. Previously, the use of bevacizumab-800CW and SGM-101 for PM detection in an open setting have been described.^{33,34} We strive to use fluorescence-guided laparoscopy to improve patient selection prior to cytoreductive surgery (CRS) and hyperthermic intraperitoneal chemotherapy (HIPEC), the only curative treatment option for colorectal PM.^{35,36} CRS + HIPEC is associated with a major incidence of morbidity up to 60% and mortality up to 5%. Furthermore, the PM 1-year recurrence rate after CRS + HIPEC is almost 40%. Therefore, selecting patients who will benefit the most – taking into account either limited palliative surgery or extensive curative-intend surgery – for this extensive treatment is crucial. Prior to CRS + HIPEC, patients undergo a diagnostic laparoscopy (DLS) to calculate the Peritoneal Carcinomatosis Index (PCI). In the Netherlands patients are suited for CRS + HIPEC with a PCI below 20. However, PCI calculation is complicated as the visual distinction between malignant, fibrotic and benign tissue without biological information is challenging. Using the tumor-specific imaging agent bevacizumab-800CW during DLS, our aim is to facilitate an easier patient selection tool prior to CRS + HIPEC with the hypothesis that fluorescence imaging can distinguish malignant and benign tissue, as has been observed previously.³³

The second part of the thesis explores new indications for optical molecular imaging. **Chapter 6 & 7** describes the clinical translation of the use of bevacizumab-800CW for a new indication namely inflammatory vascular atherosclerosis, where we aimed to identify vulnerable biological processes *ex-* and *in vivo* based on their angiogenic characteristics.

Atherosclerotic plaques prone for rupture, the so-called vulnerable plaques, are characterized by (neo-)angiogenesis mediated by VEGF, a thin fibrous cap, lipid accumulation and macrophage infiltration.³⁷ Optical imaging is a highly sensitive imaging modality to visualize one the hallmarks of the vulnerable plaque which is angiogenesis.³⁸ Due to the formation of permeable

microvasculature, endothelial dysfunction facilitates an inflammatory response by a massive cytokine release with predominantly macrophages. This frail and inflammatory situation in the vulnerable plaque leads to vessel wall thickening, intraplaque hemorrhage and eventually plaque instability.³⁹ Plaque rupture can lead to partial or total vessel occlusion and subsequent stroke.

Visualizing and treating neovascularization using angiogenesis factors like VEGF-A which induces vascularization and hyperpermeability is considered one of the holy grails in (cardio) vascular treatment. Targeting angiogenesis *in vivo* could therefore identify patients who are at risk for cardiovascular disease. Moreover, the possibility to track pathogenetic atherosclerotic specific molecular pathways opens up the possibility for drug discovery and site-specific targeting. In **chapter 6** we incubated 17 atherosclerotic plaques with 1 µg/ml bevacizumab-800CW and observed a strong correlation between high VEGF-A expression and fluorescence intensity in vulnerable plaques. We showed a positive correlation between plaque vulnerability and VEGF-A expression, macrophage infiltration and intraplaque microvessel density using immunohistochemistry. We stated that bevacizumab-800CW is capable of targeting and thereby visualizing VEGF-A overexpression in vulnerable plaques *ex vivo* and these results gave us the support to proceed with a safety / feasibility clinical trial.

In **chapter 7** we describe this translational work and the use of a novel integrated clinical optoacoustic imaging methodology into our research to visualize plaque characteristics *in vivo*. The feasibility of optoacoustic imaging for cancer detection resolving hemoglobin due to an increased angiogenesis in breast cancer and thyroid cancer has already been shown.^{40,41} Moreover, feasibility for imaging the carotid artery itself was shown in a similar fashion.⁴² In order to visualize the inflammatory process related to atherosclerosis, we included 5 healthy volunteers and 4 patients undergoing carotid endarterectomy for symptomatic carotid stenosis who received 4.5 mg bevacizumab-800CW three days prior to surgery. Unfortunately, adequate localization of bevacizumab-800CW signal in the atherosclerotic plaque was difficult due to autofluorescence related intrinsic calcifications. Optoacoustic imaging showed high hemoglobin signal *in vivo*, but no distinctive bevacizumab-800CW or lipid spectrum could be visualized due to the low quantity of tracer in the plaque and the relative deep location of the carotid artery (+/- 2-3 cm). We concluded that *in vivo* optoacoustic imaging using a low dose bevacizumab-800CW concentration was not (yet) possible. The development of dedicated optoacoustic imaging agents is required to improve non-invasive disease-targeted imaging. In order to enable this desire there are currently two compounds strategies in preclinical investigations, namely the development of organic NIR (near infrared) dyes and metallic nanoparticles. Organic dyes are safe for in-human use, but may not be sufficient for deeper-seated disease processes to be detected by optoacoustic imaging. Metallic nanoparticles offer more qualitative photophysical properties, but seem to be hepatotoxic in humans which obstructs clinical use so far.⁴³ An optimal optoacoustic agent contains a low quantum yield, a high molar extinction coefficient, a characteristic absorption spectrum with sharp peaks, high absorption in the near-infrared range and high photostability.⁴⁴ We observed the presence of bevacizumab-800CW in the atherosclerotic plaque *ex vivo* using SDS-page. The introduction of an optoacoustic compound within our fluorescence-guided imaging workflow is the next developmental step in our optical imaging program.

FUTURE PERSPECTIVES

In this thesis, we have explored multiple innovative strategies in phase I studies to eventually improve surgical decision making and patient outcome. To eventually determine the benefit for patients in subsequent phase II and III trials, standardization of outcome parameters for phase II/III studies is essential to finally implement FI in daily practice. (Inter-)national standard imaging protocols, camera systems and outcome parameters for phase II/III should be defined. Moreover, training should be provided by expertise centers. In surgical oncology, clinical parameters such as decreased incidence of local recurrence, re-operation rate or an improved 1 or 2 year morbidity can be listed as national accepted outcome parameters to determine the additional value of FGS.

This need for standardization and training is clearly relevant in non-specific ICG studies. Anastomotic leakage (AL) is a fearful complication, mostly leading to secondary surgical explorations or even life threatening intensive care admissions and mortality due to sepsis. Poor vascular perfusion is a major risk factor dysfunctioning of healthy tissue and tissue repair for example during AL of colorectal anastomosis. Therefore, early clinical detection of poorly perfused tissue is crucial to evaluate and eventually directly intervene during anastomotic surgery. Studies using ICG showed a deviation of the original surgical plan by repositioning the anastomosis up to 25%, decreasing the incidence of AL for example in esophagectomy from 24,55% to 14,08%.^{46,47} In the Netherlands, an ongoing nationwide multicenter phase III study (Netherlands Trial Register NL7502) is evaluating in 978 patients whether FI using ICG is decreasing AL in colorectal cancer surgery. However, the perfusion status in these studies is still determined by visual inspection alone. To secure objectivity, there is a need for quantification methods using for example in- and outflow parameters and fixed camera positions to overcome subjectivity and improve reproducibility.

To improve surgical decision making, the incorporation of artificial intelligence (AI), which is not described in this thesis, could be of additional value in FI. Shortly, AI tries to mimic human cognitive functions like problem solving and decision-making using algorithms with machines.⁴⁸ Computer vision is defined as adequate recognition and interpretation of patterns, videos and images. AI can thus be promising during FI and the interpretation of real-time histopathological data. The availability of large datasets allows introduction of deep neural networks which can provide the surgeon with a so-called red flag technique which characterizes regions of interest during surgery or endoscopy. Different deep learning mechanisms have already proven superiority for detection of lymph node metastasis in cancer beyond that of the human observer.⁴⁹ The first step of using AI in FI is analysis of adequate tumor delineation during histopathology combined with correlation with fluorescence-guided images, which would speed up and standardize image interpretation. International databases, consisting of clinical fluorescence images could be created to share global knowledge on image processing and will create a big data platform for FI methodology. However, these databases should be carefully developed with standardized imaging and analysis methods, so reliable AI algorithms can be tested.⁵⁰ The Response Evaluation

Criteria in Solid Tumors (RECIST), a list of standard imaging criteria to assess validity of imaging modalities concerning tumor response after diagnosis and therapy, could be such an example for the standardization of FI studies.⁵¹ Enhancing real-time clinical decision making should not overtake human interpretation, but be supportive.⁵²

In this thesis, we have shown that the use of low-dose imaging agents (antibodies and nanobodies) is able to visualize drug distribution in a variety of solid tumors. For drug development purposes, the conjugation of a fluorophore or a radionuclide to a new molecular entity of therapeutic drug could improve the development process. By testing these novel drugs in a small series of 10-20 patients, the so-called proof of concept studies, it is possible to predict biodistribution, optimal dosing and drug effectiveness and eventually reach an early point of decision making.⁵³ Using a relatively low-dose (e.g. microdose) strategy, the safety risks for patients are minimized and approval by regulatory authorities is granted.^{54,55} Microdosing is a cost- and time effective model, potentially preventing costly and non-successful phase II and III studies.

The aim of oncological surgery is complete tumor resection combined with sparing vital structure like ureters, important vessels and nerves. For example, STS dissection is mostly performed in the internervous plane, especially in the pelvis and extremities, potentially causing neurological damage and neuropathic pain. Preclinical studies investigated novel tracers to identify nerves during surgery.^{56,57} Moreover, colleagues have shown the feasibility of ureter identification during abdominal surgery using a zwitterionic fluorophore called ZW800-1.⁵⁸ Integrating these novel tracers to the tumor-specific imaging workflow has the potential to provide real-time visualization of vital structures, making surgery more precise.

We have described the use of optoacoustic imaging using high-pulsed lasers in **chapter 7**, which also can be used as a light-activator for cancer treatment. An upcoming modality is photodynamic therapy (PDT), where reactive oxygen species (ROS) are generated by photosensitizers after being excited by light irradiation using a near-infrared wavelength which eliminates cancerous tissue.⁵⁹ In almost all cancer cells, ROS promote tumor development and progression by supporting cell proliferation and homeostasis. However, an elevated endogenous ROS generation promotes anticancer activity. Using this light-based approach in a therapeutic setting, combined with the use of a tumor-specific molecular setting, therapy can be inserted with light as a driver of activation. This novel approach allows for drug activation at the location of the disease site, giving precise and personalized therapy, potentially without systemic side effects.⁵⁹ The combination of tumor visualization using switchable tracers like our pH-activated nanoprobe during surgery, combined with the release of a photosensitizer, offers a complete theranostic scheme for visualizing macroscopic and microscopic tumor tissue, and simultaneously potential ablation by PDT on demand, creating less severe systemic side-effects.

Overall conclusion

This thesis describes several critical novel steps in further improvement of disease-specific visualization by fluorescence and optoacoustic imaging in the surgical setting. We illustrate the added value of fluorescence imaging during clinical decision making during surgery. Moreover, we show the potential of bevacizumab-800CW to target the vulnerable atherosclerotic plaque with the need for the integration of innovative and novel modalities like optoacoustic imaging. In order to make our research activities more applicable in daily practice, we need to collaborate in an international framework to unify imaging agents, imaging methodology and outcome parameters. We look forward to a technical era where surgeons will be supported by enhanced imaging technologies and AI tools in a validated and standardized setting to guide surgical decision making in a more precise and adequate manner. By using illuminating solutions, surgical decision making will be eventually improved!

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CHAPTER 9

Dutch summary – Nederlandse samenvatting

NEDERLANDSE SAMENVATTING

Vaatlijden en kanker zijn de belangrijkste oorzaken van morbiditeit en mortaliteit wereldwijd. De ontwikkeling van verschillende medische beeldvormende technieken zoals echografie, CT, MRI en nucleaire modaliteiten zoals PET/SPECT heeft de afgelopen decennia geleid tot betere pre- en peroperatieve visualisatie van kanker- en vaatlijden gerelateerde aandoeningen. Mede hierdoor is een groter diagnostisch, therapeutisch en uiteindelijk chirurgisch succes bewerkstelligd. Met behulp van moleculaire beeldvorming is het mogelijk geworden om met medicijnen of zoekstoffen (tracers), gedetailleerder en nauwkeuriger ziekte-specifieke biologische karakteristieken af te beelden. Hierbij zijn nieuwe en innovatieve oncologische medicijnen ontdekt, wat heeft geleid tot een betere, ziekte-gerichte en gepersonaliseerde behandeling.¹⁻⁴ De ontwikkeling van nieuwe chemo- en immunotherapie heeft tevens bijgedragen aan een verbetering in ziekte-vrije en algehele overleving bij patiënten met verschillende typen solide tumoren.⁵ Gedurende de oncologische behandeling is een operatie een van de pijlers van de behandeling, naast radio- en chemo/immunotherapie, waarbij moleculaire beeldvorming de mogelijkheid biedt om de tumorbiologie gericht af te beelden voor en tijdens de behandeling.⁶

Het doel van een in opzet curatieve oncologische chirurgische procedure is het compleet verwijderen van de primaire tumor inclusief alle microscopische omringende tumorcellen, het zogenoemde schone snijvlak. Helaas komt het tegenwoordig bij meerdere kankersoorten nog frequent voor dat een tumor onvolledig wordt verwijderd en er dus tumorcellen op het snijvlak, de resectiemarge, achterblijven.⁷ Deze zogenaamde tumor-positieve snijvlakken leiden tot een slechtere prognose en maken aanvullende behandeling zoals een nieuwe operatie of chemo- en/of radiotherapie noodzakelijk, wat leidt tot toename van morbiditeit. Moleculaire beeldvorming biedt meerdere mogelijkheden om maligne cellen zichtbaar te maken en daarmee van benigne cellen te onderscheiden. Zo heeft tumor-specifieke nucleaire beeldvorming bewezen dat het gebruik van moleculaire informatie het diagnostische traject verbeterd en hiermee de oncologische behandeling efficiënter maakt. Middels deze beeldvormende techniek worden moleculaire tumorkarakteristieken zichtbaar, en kan tevens de interactie tussen een gelabeld medicament (radioactief of fluorescent) en de tumor gevisualiseerd worden.⁸ Het is echter, ondanks alle ontwikkelingen van de afgelopen jaren, niet mogelijk gebleken om in de oncologisch chirurgische setting beeldvormende technieken zoals CT-scans, MRI-scans en nucleaire beeldvorming van toegevoegde waarde te laten zijn in de operatiekamer.

Een veelbelovende nieuwe klinische techniek is intra-operatieve fluorescentie beeldvorming. Hierbij worden (auto-)fluorescentie signalen met specifieke fluorescentiecamera's gedetecteerd tijdens open-, laparoscopische en robot-chirurgische procedures. Momenteel moet de chirurg tijdens de operatie vertrouwen op visuele en tactiele feedback, waarbij tijdens laparoscopie en robot chirurgie ook nog de tactiele feedback ontbreekt. De toegevoegde waarde van fluorescentie beeldvorming voor het verbeteren van anatomische en biologische informatie kan meerwaarde bieden tijdens de procedures op basis van de inherente eigenschappen van optische beeldvorming. Door gebruik te maken van tumor-specifieke tracers, welke accumuleren in tumorcellen of lymfkliermetastasen, kan de specificiteit met beeldvorming in hoge resolutie

opgeschroefd worden. Kwalitatieve en kwantitatieve fluorescentie beeldvorming vindt plaats met hoge resolutie.^{9,10} Vergeleken met nucleaire beeldvormende technieken is fluorescentie goedkoper en is er geen sprake van radioactiviteit, waardoor de belasting voor zowel de patiënt als het aanwezige personeel minimaal is. Bovendien behoeft fluorescentie beeldvorming, in tegenstelling tot de nucleaire apparatuur, weinig fysieke ruimte waardoor het makkelijker is om te implementeren in de operatiekamers. Tumor-specifieke fluorescentie beeldvorming heeft de afgelopen jaren significant aan wetenschappelijke en klinische interesse gewonnen, waarbij de meest studies binnen dit onderwerp zich richten op de ziektebiologie van tumor-specifieke eiwitten.¹¹⁻¹³ Het uiteindelijke doel is om de zichtbaarheid van tumoren en uitzaaiingen te verbeteren en hiermee een nieuwe standaard voor tumorvisualisatie neer te zetten ter faveure van de uitkomst bij patiënten met solide tumoren.¹⁴

Fluorescentie beeldvorming, bijvoorbeeld met de sinds 1971 goedgekeurde fluorescente tracer indocyanine groen (ICG), wordt wereldwijd al gebruikt om doorbloeding en de aanwezigheid van tumorcellen te visualiseren.^{15,16} Adequate doorbloeding is essentieel voor wondgenezing. Slechte doorbloeding is een grote risicofactor voor disfunctioneren van gezond weefsel en weefselreparatie zoals het geval kan zijn bij naadlekkage van darmanastomosen; een gevreesde complicatie waarbij meestal een nieuwe operatie noodzakelijk is en zelfs levensbedreigende situaties ten gevolge van sepsis kunnen ontstaan. Vroege detectie van slecht doorbloed weefsel zou dus cruciaal kunnen zijn om de aangelegde anastomose te evalueren en eventueel al tijdens de operatie te kunnen interveniëren. Studies gebruik makend van ICG tonen aan dat het chirurgische plan vergeleken met de standaard zorg tot 25% veranderd op basis van fluorescentie beeldvorming, waarbij de geplande locatie van de anastomose aangepast wordt op basis van het fluorescentie signaal. Bij slokdarmresecties daalde hierbij de incidentie van naadlekkages zelfs van 24,55 % naar 14,08%. Toch is er nog steeds geen consensus over de dosering van ICG, het tijdstip van beeldvorming na injectie en de kwantificatie van ICG-uptake waardoor deze studie uitkomsten mogelijk zeer heterogeen en lastig te vergelijken zullen zijn. Daardoor worden beslissingen momenteel nog gemaakt op subjectieve bevindingen en interpretaties van de vaak niet-optimaal getrainde chirurg wat een grote kans op observer-bias geeft. Het specifieke karakter van ICG en het gebrek aan standaardisatie maakt het gebruik tijdens oncologische chirurgie uitdagend, maar haalbaar.

Verbetering van beeldvormende technieken is ook binnen de cardiovasculaire tak binnen de heekunde relevant. Jaarlijks overlijden 17.9 miljoen mensen aan hart- en vaatziekten, en deze aandoeningen zijn dan ook de grootste doodsoorzaak wereldwijd.¹⁷ Duplex echografie is bij vaatlijden vaak de eerste non-invasieve beeldvormende keuze.¹⁸ CT-angiografie en MRI-angiografie kunnen atherosclerose zichtbaar maken, maar bieden beiden een relatief lage resolutie en zijn gevoelig voor beeldvormingsartefacten, wat de uiteindelijke interpretatie bemoeilijkt. Moleculaire informatie omtrent vaatnieuwvorming (angiogenese) en vaathervorming van atherosclerotische plaques kan mogelijk nieuwe inzichten geven in het visualiseren van de zogenaamde "kwetsbare" atherosclerotische plaques.¹⁹ Deze kwetsbare plaques zijn berucht om hun verhoogde risico op ischemische hersen beroertes, cardiale ischemie en perifeer vaatlijden.

Vroege detectie met behulp van nieuwe beeldvormende technieken is derhalve van belang om patiënten eerder te diagnosticeren en zo nodig te behandelen.

De implementatie van fluorescentie beeldvorming binnen de vaatchirurgie is wenselijk maar lastig, aangezien de penetratiediepte van optische beeldvorming (+/- 1 centimeter) beperkt is. Optoakoestische beeldvorming, een innovatieve niet-invasieve beeldvormende techniek, kan dit verbeteren. Het combineert echografie met optische illuminatie door gebruik te maken van pulsatie laserlicht. Het laserlicht wordt op specifieke golflengtes geabsorbeerd door moleculen, welke deze energie omzetten naar geluidsgolven op een vooraf in te stellen en te detecteren golflengte. Deze techniek kan zowel endogene contrastmiddelen (bijvoorbeeld hemoglobine, melanine en lipiden) als exogene tracers detecteren tot een diepte van 5 centimeter. Meerdere vroege-fase klinische studies hebben reeds de voordelen van endogene optoakoestische beeldvorming binnen de oncologie en vaatziekten laten zien.^{20,21}

Het doel van dit proefschrift is om inzicht te krijgen in de veiligheid en de *in-* en *ex vivo* effectiviteit van nieuwe fluorescente tracers en om daarbij een gestandaardiseerde workflow te creëren voor verschillende tumortypes (weke delen tumoren, hoofd-hals kanker, borstkanker, darmkanker en slokdarmkanker). Hiermee richten we ons op een toekomst waarin de chirurgische beslisvorming direct peroperatief verbeterd kan worden met behulp van fluorescentie beeldvorming. Daarnaast evalueren we de mogelijkheid om kwetsbare atherosclerotische plaques beter te visualiseren door gebruik te maken van het fluorescent gelabelde antilichaam bevacizumab-800CW en optoakoestische beeldvorming.

Hoofdstuk 1 geeft een algemene introductie over optische en optoakoestische beeldvorming en de uiteenzetting van dit proefschrift.

Het eerste gedeelte van dit proefschrift beschrijft de vooruitgang binnen fluorescentie-geleide chirurgie. In **hoofdstuk 2** wordt de overexpressie van het eiwit vascular endothelial growth factor-A (VEGF-A) gebruikt als target voor fluorescentie beeldvorming.^{22,23} We laten zien dat de tracer bevacizumab-800CW weke delen tumoren *in-* en *ex vivo* kan visualiseren. De meeste weke delen tumoren, maar met name myxofibrosarcomen, zijn berucht vanwege hun hoge aantal tumor-positieve snijvlakken (10-35%).²⁴ Dit zorgt voor een hoger aantal recidieven en een toename van morbiditeit en daling in kwaliteit van leven. Recent is er een fluorescentie studie verschenen die de aspecifieke tracer ICG gebruikt om tumordetectie in sarcomen te bevorderen.²⁵ Hierin wordt beschreven dat tumor-visualisatie mogelijk is, maar wordt geen tumorspecificiteit aangetoond. Tevens is er één preklinische studie en één proof-of-concept studie met één patiënt beschreven waarin het affibody ABY-029 (met als target endothelial growth factor receptor, EGFR) de weke delen tumor op laat lichten.²⁶ EGFR lijkt echter niet het meest optimale en adequate target, aangezien met name myxofibrosarcomen een hoge expressie van tumor endothelial marker 1 (TEM1) en VEGF-A tonen, met juist lagere EGFR expressie.²³ Aangezien EGFR ook tot expressie komt in omliggend benigne weefsel geeft dit derhalve waarschijnlijk geen goede contrastintensiteit. Wij laten zien dat beeldvorming van

weke delen tumoren met intraveneus toegediende bevacizumab-800CW (10 mg flat-dose bolus injectie) een *in vivo* tumor-to-background ratio (TBR) van 2.0 en *ex vivo* een TBR van 2.67 geeft. Daarbij zijn geen tracer-gerelateerde bijwerkingen geobserveerd in alle doseringsgroepen (10, 25 en 50 mg bolus injectie). We concluderen dat analyse van het resectievlak bij weke delen sarcomen direct na de chirurgische excisie de potentie heeft om de chirurg te begeleiden gedurende de intra-operatieve beslisvorming. De data van deze studie illustreerden heel duidelijk dat een snijvlak met hoge fluorescentie-intensiteit duidt op een positief snijvlak. Identificatie van deze positieve snijvlakken *ex vivo* (ook wel back-table imaging genoemd), hetgeen binnen enkele minuten na excisie verricht kan worden, kan de chirurg in de toekomst doen besluiten tot een direct aanvullende resectie. Onze resultaten zullen worden gebruikt voor de opvolgende fase II studie met als uiteindelijke doel om het aantal tumor-positieve snijvlakken in deze zeldzame tumorgroep terug te dringen en daarmee morbiditeit en aanvullende chirurgische interventies te voorkomen.

Weke delen tumoren liggen meestal in een gebied met vele neurovasculaire verbindingen. De identificatie en het chirurgisch sparen van deze vitale structuren is daarbij van groot belang om morbiditeit zoals neuropathische pijn te voorkomen. Er wordt veel onderzoek gedaan naar nieuwe tracers die het mogelijk maken om necrose, zenuwen, vaten en ureters te visualiseren.²⁷ Het gebruik van deze tracers in combinatie met tumor-specifieke tracers (zoals bevacizumab-800CW) tijdens oncologische chirurgie van weke delen tumoren zou kunnen leiden tot reseceren van tumorcellen waarbij vitale structuren gespaard blijven. Om klinische implementatie van deze innovatieve fluorescentie technieken te bewerkstelligen, zijn we afhankelijk van de gebruikte uitkomstparameters van fase II en III klinische studies. Binnen de oncologische chirurgie zou het klinisch van meerwaarde zijn om te achterhalen of fluorescentie uiteindelijk leidt tot een verminderde incidentie van tumorrecidieven of aanvullende resecties. Ook daling in morbiditeit kan gebruikt worden als nationaal geaccepteerde parameter om de toegevoegde waarde van intra-operatieve fluorescentie beeldvorming te bepalen.²⁸

In **hoofdstuk 3** beschrijven we de veiligheid en mogelijkheid van tumor generieke beeldvorming in vier verschillende kankersoorten (hoofd-halskanker, borstkanker, slokdarmkanker en darmkanker). Want ondanks positieve resultaten met moleculaire tumormarkers kleven er ook nadelen aan de tumor-specifieke benadering met monoklonale antilichamen zoals bevacizumab-800CW. Vanwege de diversiteit in oncologische geno- en fenotypen tussen verschillende kankersoorten, zijn de tracers (nog) niet breed toepasbaar bij verschillende type tumoren, en zelfs binnen één tumortype.²⁹ Een methode om deze heterogeniteit van tumorcellen te omzeilen is door gebruik te maken van generieke eigenschappen van alle tumorcellen, zoals het zure extracellulaire milieu.³⁰ Deze extracellulaire acidose wordt veroorzaakt door anaerobe glycolyse en tumor hypoxie in alle solide tumoren.³¹ ONM-100 maakt gebruik van deze 'hallmark of cancer' en is een nieuwe zogenaamde "smart-activatable" fluorescente tracer welke bestaat uit pH-gevoelige micellen gelabeld met ICG. De micellen dissociëren in het zure extracellulaire milieu en zorgen derhalve voor tumor-specifieke labeling (zogenaamde nanoscale macromolecular instability).³² We laten zien dat ONM-100 veilig is in alle doseringsgroepen (0.1 tot 1.2 mg/kg

ONM-100). Bovenal wordt een zeer scherpomlijnd tumor-specifiek fluorescentie signaal gezien in alle tumortypes en worden alle tumor-positieve marges in beeld gebracht middels *ex vivo* en wondbed beeldvorming (9/9, 100% sensitiviteit). We vinden intra-operatief 4 occulte laesies en daarnaast additionele (pre)maligne laesies tijdens de histopathologische verwerking middels fluorescentie beeldvorming, welke initieel gemist werden.

Het voordeel van de tracer ONM-100 ten opzichte van andere tracers is dat het maximaal 1 dag preoperatief toegediend kan worden, wat implementatie in de klinische praktijk makkelijker maakt. In de lopende fase II studie (www.clinicaltrials.gov: NCT03735680) wordt onderzocht of de tracer nog korter voor de operatie gegeven kan worden, en of deze ook toepasbaar is bij andere kankersoorten zoals ovariumkanker en prostaatkanker. Door gebruik te maken van generieke tumoreigenschappen kan ONM-100 potentieel beschouwd worden als een “one-size-fits-all” oplossing.

Door gebruik te maken van fluorescente tracers kan het weefsel direct na excisie van de tumor beoordeeld worden door de chirurg. Een gestandaardiseerde setting kan hierin bijdragen aan adequate sampling van positieve snijvlakken door evaluatie van het weefsel en sampling van de weefsellamellen direct na de excisie, hetgeen “intraoperative pathology assisted surgery (IPAS)” wordt genoemd. IPAS kan daarbij direct invloed hebben op de operatie, aangezien de informatie binnen enkele minuten na de excisie verkregen kan worden en er eventueel direct een nieuwe resectie uitgevoerd kan worden op basis van een combinatie van klinische interpretatie en het intra-operatief verkregen beeldmateriaal. Het voordeel van IPAS ten opzichte van intra-operatieve beeldvorming is de gestandaardiseerde setting, waarin nadelen als sterilisatie, de afstand tussen de camera en weefsel en de aanwezigheid van omgevingsfactoren (licht) omzeild kunnen worden.³³ Door gebruik te maken van efficiënte protocollen kan IPAS een potentiële aanvulling zijn op de standaard tactiele en visuele informatie die de chirurg heeft.

Detectie van tumor-positieve marges is hoofdzakelijk subjectief aangezien er nog geen gestandaardiseerde (inter)nationale beeldvormende optische imaging protocollen zijn. In **hoofdstuk 4** beschrijven we het gebruik van IPAS bij patiënten met hoofd-hals kanker die preoperatief ONM-100 hebben gekregen. Door het binaire uit/aan principe van de tracer kan een scherpe omlijning tussen maligne en benigne weefsel zichtbaar worden gemaakt. We gebruiken de TBR met een afkappunt > 1.5 om te evalueren welk snijvlak verdacht is voor een positieve marge en zien dat we daarmee alle tumor-positieve marges snel na de ingreep kunnen visualiseren.

In **hoofdstuk 5** beschrijven we een nieuwe indicatie voor fluorescentie-geleide chirurgie binnen de patiëntengroep met colorectale peritoneaal metastasen. De enige curatieve behandeling voor deze patiënten is cytoreductieve chirurgie (CRS) en hypertherme intraperitoneale chemotherapie (HIPEC), een behandeling met een hoge morbiditeit (tot 60%) en mortaliteit (tot 5%).³⁴ Daarbij is de 1-jaars recidief kans na CRS+HIPEC rond de 40%. In de huidige tijd ondergaan patiënten bij de bepaling van het behandeltraject eerst een diagnostische

laparoscopie om de peritoneale kanker index (PCI) te bepalen. Patiënten met een PCI onder de 20 worden in Nederland als geschikte kandidaten bestempeld voor CRS+HIPEC. Het betrouwbaar onderscheid maken tussen maligne en benigne weefsel (zoals fibrose) tijdens de laparoscopie is lastig, en hier biedt fluorescentie mogelijk uitkomst. Er is reeds onderzoek gedaan naar het gebruik van bevacizumab-800CW en SGM-101 (target carcino embryonaal antigeen).^{35,36} Wij zullen in het UMCG onderzoeken of de integratie van fluorescentie beeldvorming bij de diagnostische laparoscopie zorgt voor een betere patiëntselectie voor CRS+HIPEC. Daarbij verwachten we dat fluorescentie beeldvorming een vermindering van de PCI score geeft en derhalve de selectie voor CRS+HIPEC kan verbeteren en vergroten.

Het tweede gedeelte van dit proefschrift exploreert nieuwe indicaties voor moleculaire beeldvorming. **Hoofdstuk 6 & 7** beschrijven de klinische translatie van het gebruik van bevacizumab-800CW, waarbij we proberen om kwetsbare atherosclerotische plaques te visualiseren, met als target biologische processen gericht op angiogenese.

Kwetsbare atherosclerotische plaques worden gekarakteriseerd door de aanwezigheid van een hoge mate van neo-angiogenese met een belangrijke rol voor VEGF, een grote hoeveelheid lipiden, een dunne fibreuze rand en de infiltratie van macrofagen.³⁷ Door de vorming van permeabele microvasculatuur en daarbij disfunctioneren van het vaatendotheel wordt een inflammatoire respons uitgelokt, waarbij een cytokinestorm met voornamelijk macrofagen tot stand komt.³⁸ Dit kwetsbare en inflammatoire milieu leidt tot vaatwandverdikking, instabiliteit van de plaque en risico op bloeding in de plaque. Het scheuren van de plaque kan leiden tot partiële of totale vaatafsluiting en distale embolisatie (in de volksmond beroerte genoemd).

Het vroegtijdig screenen op neovascularisatie, bijvoorbeeld door het visualiseren van VEGF-A *in vivo*, zou mogelijk patiënten kunnen identificeren die een verhoogd risico lopen op een beroerte. In **hoofdstuk 6** beoordelen we 17 atherosclerotische plaques met 1 µg/ml bevacizumab-800CW, waarbij we een sterke correlatie tussen een hoge VEGF-A expressie en fluorescentie intensiteit in kwetsbare plaques zien. We laten een evidente correlatie zien met behulp van immunohistochemische kleuringen tussen kwetsbaarheid van de plaques en VEGF-A expressie, macrofaag infiltratie en de densiteit van microvasculatuur. We concluderen dan ook dat bevacizumab-800CW een geschikte tracer is om VEGF-A overexpressie aan te tonen bij kwetsbare plaques. Deze bevindingen hebben geresulteerd in de totstandkoming van **hoofdstuk 7**.

In **hoofdstuk 7** beschrijven we het translationele werk met het gebruik van optoakoestische beeldvorming om de kwetsbare plaque te visualiseren. Meerdere studies hebben laten zien dat optoakoestische beeldvorming voor tumordetectie werkt door het opvangen van hemoglobinesignalen.^{20,39,40} Daarbij hebben collega's reeds laten zien dat 3D beeldvorming van de arteria carotis mogelijk was.⁴¹ Om het inflammatoire proces, dat gerelateerd is aan atherosclerose, weer te geven zijn 5 gezonde vrijwilligers en 4 patiënten met een symptomatische carotisstenose geïnccludeerd. De symptomatische patiënten ondergaan een carotidesobstructie en krijgen 3 dagen preoperatief een microdosering van 4.5 mg bevacizumab-800CW. Helaas blijkt adequate

visualisatie van deze lage dosering bevacizumab-800CW in de atherosclerotische plaque met optoakoestische beeldvorming niet mogelijk gezien de diepteligging en lage hoeveelheid tracer in de plaque. Echter, *ex vivo* is wel aanwezigheid van dat bevacizumab-800CW aangetoond in de atherosclerotische plaque. De verdere ontwikkeling van speciale en vooral optoakoestisch efficiënte tracers is zinvol om non-invasief ziekte specifieke en diepere optoakoestische beeldvorming mogelijk te maken. Er zijn hedendaags twee routes die preklinisch onderzocht worden: I) het gebruik van organische nabij-infrarode kleurstoffen en II) het gebruik van metalen nanopartikels.⁴² Organische kleurstoffen zijn veilig voor humaan gebruik, maar zijn waarschijnlijk niet bruikbaar voor dieper gelegen ziektes. Metalen nanopartikels hebben betere fotofysische eigenschappen, maar zijn waarschijnlijk humaan hepatotoxisch wat de klinische translatie in de weg staat.⁴³ Het toevoegen van een specifieke optoakoestische tracer aan onze fluorescentie workflow is de volgende ontwikkelingsstap in ons onderzoeksprogramma.

ALGEMENE CONCLUSIE

Dit proefschrift beschrijft meerdere innovatieve routes naar verbetering van ziekte-specifieke visualisatie door middel van fluorescentie en optoakoestische beeldvorming in de chirurgische setting. We laten de toegevoegde waarde van fluorescentie beeldvorming zien gedurende de chirurgische klinische beslisvorming. Daarbij benadrukken we de behoefte aan standaardisatie en integratie van deze nieuwe technieken in de standaard imaging workflow. We kijken uit naar een toekomst waarin chirurgen ondersteund worden door vooruitstrevende beeldvormende technieken in een gevalideerde en gestandaardiseerde setting. Door lichtgevende oplossingen zal de chirurgische beslisvorming uiteindelijk preciezer en beter worden!

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CHAPTER 10

Appendices

List of publications

Dankwoord

Curriculum Vitae

LIST OF PUBLICATIONS

F.J. Voskuil, S.J. de Jongh, W.T.R. Hooghiemstra, M.D. Linssen, **P.J. Steinkamp**, et al. Fluorescence-guided imaging for resection margin evaluation in head and neck cancer patients using cetuximab-800CW: A quantitative dose-escalation study.

Theranostics, 2020

P.J. Steinkamp*, F.J. Voskuil*, T. Zhao , et al. Exploiting metabolic acidosis in solid cancers using a tumor-agnostic novel pH-activatable nanoprobe for clinical fluorescence-guided surgery.

Nature Communications, 2020

P.J. Steinkamp*, F.J. Voskuil, M.J.H. Witjes, G.M. van Dam. Behind the paper: A novel pH-sensitive fluorescent tracer for the detection of a variety of solid tumors. A great journey with interesting results!

Nature Community Research, 2020

P.J. Steinkamp*, B.K. Pranger*, M. Li, et al. Fluorescence-guided visualization of soft tissue sarcomas by targeting vascular endothelial growth factor: a phase 1 single-center clinical trial.

Journal of Nuclear Medicine, 2020

COVIDSurg Collaborative (o.a. **P.J. Steinkamp**). Mortality and pulmonary complications in patients undergoing surgery with perioperative SARS-CoV-2 infection: an international cohort study.

The Lancet, 2020

P.J. Steinkamp*, P.K.C. Jonker, * W.Y. van der Plas*, et al. Perioperative mortality, pulmonary complications and thromboembolic events in patients undergoing elective or emergency surgery during the COVID-19 pandemic: a Dutch multicenter matched-cohort clinical study.

Surgery, 2020

P.J. Steinkamp*, F.A. van der Zant*, J.E.K.R. Hentzen, G.M. van Dam, S. Kruijff. Improving visualization of colorectal metastatic cancer using laparoscopic fluorescence-guided surgery.

Medical Research Archives, 2020

L.A. Huisman, **P.J. Steinkamp**, J.L. Hillebrands, et al. Bevacizumab-800CW: ex vivo fluorescence imaging of angiogenesis in (non-) culprit human carotid atherosclerotic plaques.

Scientific Reports, 2021

F.B. Poelmann, T. Koëter, **P.J. Steinkamp**, et al. The immediate impact of the COVID-19 pandemic on burn-out, work-engagement and surgical training in the Netherlands.

Surgery, 2021

P.J. Steinkamp*, F.J. Voskuil*, T. Zhao , et al. A standardized framework for fluorescence-guided margin assessment for head and neck cancer using a tumor acidosis sensitive optical imaging agent

Molecular Imaging & Biology, 2021

P.J. Steinkamp*, F.J. Voskuil*, B. van der Vegt, et al. Intra-operatieve visualisatie van verschillende soorten solide tumoren middels een pH-geactiveerde fluorescente tracer.

Nederlands Tijdschrift voor Heelkunde, 2021

P.J. Steinkamp*, J. Vonk*, L.A. Huisman, et al. VEGF-targeted multispectral optoacoustic tomography and fluorescence molecular imaging in human carotid atherosclerotic plaques

Diagnostics, 2021

P.J. Steinkamp*, S.J. de Jongh*, M. Lopez Alvarez, et al. Esophageal neoplasia detection using fluorescently labeled lectins

Submitted

COVIDSurg Collaborative (o.a. **P.J. Steinkamp**) Machine learning risk prediction of mortality for patients undergoing surgery with perioperative SARS-CoV-2: the COVIDSurg mortality score.

British Journal of Surgery, 2021

* Both authors contributed equally

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Het was in 2020 niet de bedoeling om het lijdend voorwerp van een UMCG ochtendoverdracht te zijn. Maar soms kop je in april een Volvo wat het levensperspectief enigszins omgooit. Mijn enorme waardering voor de spoedeisende hulp van het UMCG en de afdeling Neurologie voor de uitmuntende hulp, ook namens mijn ouders! Fijn dat ik de eerste dagen op de medium care mijn fietsbroek aan mocht houden terwijl ik 's nachts boos op de gang zat. Respect voor alle collega's en vrienden die zéér "coronaproof" en tot frustratie van de verpleging op afdeling K2 in witte jas langskwamen, soms zelfs met planten, naambordjes om de neurologische onderzoeken te boycotten en frikandellen speciaal. De hulp en interesse van velen heeft mij door deze bijzondere en achteraf leerzame periode gesleept.

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CURRICULUM VITAE

Pieter Jan Steinkamp werd op 20 februari 1991 in Eibergen geboren. Hij groeide aldaar op met zijn ouders Bas en Liesbeth en zijn zus Fieke. Hij doorliep tot 2009 het gymnasium van Het Assink Lyceum in Eibergen & Haaksbergen.

Pieter studeerde geneeskunde aan de Rijksuniversiteit Groningen tussen 2009 en 2016. Gedurende de studie was hij actief in verschillende (sport)commissies en was hij voorzitter van Stichting Sociëteit Volonté. Zijn wetenschappelijke stage vond voorafgaand aan de masterfase plaats op de afdeling orthopedie onder leiding van prof. dr. S. Bulstra. Tijdens zijn master voltooide hij zijn coschappen in het UMCG en het Sint Elisabeth Hospitaal in Willemstad op Curaçao, met verdiepende coschappen op de afdelingen orthopedie en chirurgie van het Martini Ziekenhuis. In januari 2017 startte hij als ANIOS chirurgie in het Martini Ziekenhuis bij opleider dr. W. Kelder. Na een leuk en leerzaam jaar startte Pieter zijn promotietraject in het UMCG onder leiding van prof. dr. G.M. van Dam, prof. dr. S. Kruijff en prof. dr. M.J.H. Witjes met als hoofdonderwerp optische en optoacoustische beeldvorming binnen de oncologische chirurgie en de vaatchirurgie. Hij leidde meerdere klinische studies en presenteerde zijn resultaten op verschillende grote internationale congressen. Daarnaast werkte hij gedurende zijn promotietraject als basisarts voor Zorggroep Groningen, nam hij plaats in meerdere commissies en is hij voorzitter van het Symposium Experimenteel Onderzoek Heelkundige Specialismen 2021.

Pieter is sinds 2021 ANIOS chirurgie in het UMCG bij opleiders dr. R.J. van Ginkel en dr. J.F.M. Lange. Hij woont samen met Hester Witteveen in Groningen.