Intraoperative detection of tumor tissue in peritoneal carcinomatosis of colorectal origin using a VEGF-targeted Optical Fluorescent Imaging Tracer

A single centre pilot study (HI-LIGHT study NL45588)

Clinical Study Protocol

Authors: University Medical Center Groningen (UMCG)

Version: 3.0

Date: February 28, 2014

Short title: VEGF-Targeted Near-Infrared Fluorescence imaging in Peritoneal Carcinomatosis

Protocol IDs: **CCMO-NL-45588**

EudraCT number 2013-003066-14

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LIST OF ABBREVIATIONS AND RELEVANT DEFINITIONS

ABR	ABR form, General Assessment and Registration form, is the application form that is required for submission to the accredited Ethics Committee (in Dutch: ABR = Algemene Beoordeling en Registratie)
AR	Adverse Reaction
CA	Competent Authority
CCMO	Central Committee on Research Involving Human Subjects (in Dutch: Centrale Commissie Mensgebonden Onderzoek)
CRF	Case Record Form
CTCAE	Common Terminology Criteria for Adverse Events, a grading system to record adverse events
CV	Curriculum Vitae
DSMB	Data Safety Monitoring Board
EU	European Union
EudraCT	European drug regulatory affairs Clinical Trials
FMT	Fluorescence Molecular Tomography
GCP	Good Clinical Practice
GMF	Good Manufacturing Practice
HIPEC	Hyperthermic IntraPEritoneal Chemotherapy
IB	Investigator's Brochure
IC	Informed Consent
IHC	Immunohistochemistry
IMP	Investigational Medicinal Product
IMPD	Investigational Medicinal Product Dossier
METC	Medical research ethics committee (MREC); in Dutch: medisch ethische toetsing commissie (METC)
NIR	Near-infrared

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PC	Peritoneal Carcinomatosis
PCI	Peritoneal Carcinomatosis Index
PI	Principal Investigator
(S)AE	(Serious) Adverse Event
SD	Standard deviation
SPC	Summary of Product Characteristics (in Dutch: officiële productinfomatie IB1-tekst)
Sponsor	The sponsor is the party that commissions the organisation or performance of the research, for example a pharmaceutical company, academic hospital, scientific organisation or investigator. A party that provides funding for a study but does not commission is not regarded as the sponsor, but referred to as a subsidising party.
SUSAR	Suspected Unexpected Serious Adverse Reaction
TBR	Tumor-to-Background Ratio
UMC	University Medical Center / Universitair Medisch Centrum
VEGF	Vascular Endothelial Growth Factor
Wbp	Personal Data Protection Act (in Dutch: Wet bescherming persoonsgevens)
WMO	Medical Research Involving Human Subjects Act (in Dutch: Wet Medisch-wetenschappelijk Onderzoek met mensen)

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Summary

Rationale:
This project consists of the realization and clinical validation of intraoperative imaging of tumor tissue in the case of peritoneal carcinomatosis (PC) of colorectal origin. Currently, it is not possible to determine the microscopic extent of peritoneal dissemination of cancer during surgery other than by the naked eye and manual inspection. The decision whether or not a patient could benefit from surgery or whether the disease can be deemed as resectable or not, is based on the impression of the visual and manual inspection of the surgeon. It seems reasonable that by resecting microscopic in addition to macroscopic disease, the number of R0 resections will increase and outcome will improve. By applying a method to assess the extent of peritoneal dissemination of cancer through a novel targeted optical fluorescent imaging methodology, both the staging and the resection will be more optimal.
VEGF-A (Vascular Endothelial Growth Factor – A) is highly upregulated in tumor tissue of patients with PC of colorectal origin (own UMCG data set: (n=35), 100%) and can be targeted by using the VEGF antibody Bevacizumab (Avastin).
The objective of the proposed study is to determine the diagnostic accuracy of intraoperative detection of tumor tissue of peritoneal carcinomatosis of colorectal origin by using a near-infrared fluorophore, 800CW, conjugated to bevacizumab resulting in a bevacizumab-IRDye800CW imaging compound, administered at micro dose levels (i.e. 30 nmol, or 4,5 mg). The compound has been shown to be safe at a microdosing regimen in an earlier phase I clinical study in patients with breast cancer executed at the UMCG.
Medical and surgical oncologists, pharmacists, chemists, and molecular biologists experienced in carrying out clinical translational studies using bevacizumab-IRDye800CW are involved in this project.

Objective:
Determining the feasibility and diagnostic accuracy in terms of sensitivity and specificity of intraoperative detection of peritoneal carcinomatosis of colorectal origin by intraoperative fluorescence imaging using the VEGF-targeting optical imaging agent bevacizumab-IRDye800CW in comparison to standard histology. Ex vivo immunohistochemical analyses and fluorescence microscopy will be used to confirm the presence of VEGF-A and bevacizumab-IRDye800CW in excised tumor tissue.

Study design:
Interventional pilot study: non-randomized, open label, uncontrolled with single group assignment.
The new VEGF-targeting fluorescent tracer (bevacizumab-IRDye800CW) will be administered intravenously two days before the surgical procedure is scheduled (procedure at day 3).

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During the imaging procedure we will compare the peritoneal cancer index (PCI) that were identified using bevacizumab-IRDye800CW with the standard PCI as determined by visual and tactile inspection by the surgeon.
After the surgical cytoreduction biopsies will be taken separately from areas with fluorescent and no-fluorescent spots during epi-illumination for *ex vivo* analyses (totalling 5 fluorescent vs 5 non-fluorescent). These samples will be used to determine the sensitivity and specificity using H&E pathology as determined by a pathologist blinded for the fluorescent imaging results.
Subsequently, the NIR fluorescent signal of different lesions will be quantified ex vivo (off-table).
In addition, video registration will be performed of parts of the imaging procedure. Biopsies will be analysed (described below).
The intraoperative imaging procedure will be carried out at the University Medical Center Groningen, Department of Surgery.

Study population:
Ten patients scheduled for cytoreductive surgery followed by hyperthermic intraperitoneal chemotherapy (HIPEC) for peritoneal carcinomatosis of colorectal cancer will undergo intraoperative near-infrared fluorescence imaging with the VEGF-targeted optical imaging agent bevacizumab-IRDye800CW.

Intervention (if applicable):
Patients scheduled for a HIPEC procedure for peritoneal carcinomatosis of colorectal origin will be consented for this study. There will be three study related visits. During a screening visit (visit 1), eligibility will be evaluated and patient characteristics will be collected. During the second visit 4.5 mg of bevacizumab-IRDye800CW will be administered intravenously. The patient will then be observed for 1 hour post administration. One day after administration of the tracer (visit 3 one day before surgery) the patient is administered to the hospital as in the standard procedure, or the patient can stay after the tracer injection if this more convenient for the patient. During the HIPEC procedure the fluorescent imaging will be performed and data acquired.

	Screening Visit	Tracer injection	Operation date
Patients with peritoneal carcinomatosis from colorectal origin	At Inclusion	2 days prior to surgery	Intraoperative Multispectral Fluorescence Reflectance Imaging (MFRI)

Study Aims:

Determination of sensitivity ands specificity of the sampled (non-) fluorescent tissue acquired intraoperatively compared to standard histopathological examination for the presence or absence of tumor tissue. (10 samples per patient).

Determining the detection rate of tumor tissue by calculating the PCI with and without fluorescence imaging.

Localization and (semi) quantification of a fluorescent signal of tumor tissue and surrounding tissue during NIR fluorescence imaging ex vivo.

Collection of safety data of bevacizumab-IRDye800CW

In vivo NIR fluorescence quantification by post-processing vs. *ex vivo* VEGF levels in biopsies and additional *ex vivo* analyses (using immunohistochemistry (IHC).

Nature and extent of the burden and risks associated with participation, benefit and group relatedness:
In this study, safety data related to (the administration of) the tracer will be collected and evaluated. Based on clinical experience in the first thirteen breast cancer patients (NL37479.042.11) and three rectum carcinoma patient in the RAPIDO trial (NL36315.042.11), animal toxicity studies and the fact that we will administrate a low, non-therapeutic (single dose 4.5 mg bevacizumab-IRDye800CW vs 5 mg/kg bevacizumab in therapeutics), no adverse events are expected following administration of bevacizumab-IRDye800CW. In the first thirteen breast cancer patients, included in NL37479.042.11, and three rectum carcinoma patient, included the RAPIDO trial (NL36315.042.11), no toxicity and adverse reaction were observed. To assess more information regarding the safety of bevacizumab-IRDye800CW in the current study, safety data will be collected comparable as done in NL37479.042.11 and NL36315.042.11. The investigators will have close contact with the (same) investigators of NL37479.042.11 and NL36315.042.11 regarding safety data related to bevacizumab-IRDye800CW, collected in both studies.
In the current protocol, patients will undergo the HIPEC procedure with additional fluorescence imaging of the tumour spots found during the debulking procedure. The imaging procedure will add a maximum of 30-45 minutes of operation time.
The time investment of the subjects is considered reasonable. The procedures at the screening visit and the tracer administration visit will take around 4 hours total.

1. Introduction and rationale

1.1 Epidemiology

Peritoneal carcinomatosis (PC; peritoneal dissemination of cancer) is a frequent form of end-stage colorectal carcinoma. This form of dissemination is associated with aggressive tumor growth and poor prognosis. The median survival of untreated peritoneal carcinomatosis is 22 weeks in colorectal cancer (Oncoline Nederland (www.oncoline.nl)).
After treatment (cytoreduction and HIPEC) the survival improves to a median of 2 years. 20% of the patients live longer than 5 years and are probably cured. The 80% develops recurrence of disease with a median time to recurrence of 5-14 month depending on the completeness of cytoreduction.[1]

1.2 Treatment

In selected cases of PC of colorectal origin cytoreduction in combination with a HIPEC-procedure (hyperthermic intraperitoneal chemotherapy) is performed. In this procedure, cytoreduction is followed by flushing the abdomen with heated chemotherapy. With this treatment the median survival in colorectal cancer improves to 21 months and the 5-year survival improves to about 30%.[2]

In one third of the cases, radical cytoreduction is not possible because vital abdominal organs are involved or the tumor-load is too extensive. In these patients the abdomen is closed without resection and no further surgery or HIPEC is performed. Unfortunately, it is not possible to reliably predict pre-operatively whether the tumor tissue will be resectable. The imaging modality of first-choice is a CT-scan, which is currently the most sensitive way to determine the extent of PC pre-operatively, with its inherent limitations of not reliably predicting the total tumor burden and its specific location due to limited specificity and resolution.

1.3 Need for tumor specific imaging

As for intra-operative imaging, it is important to realize that unintentionally leaving residual disease is a factor that is partly in the hands of the surgeon. Currently, the surgeon can only obtain visual and tactile information by using his/her eyes and hands during the operation. Considering the fact that the rate of positive margins is still substantial as remaining tumor tissue, hands and eyes are clearly not sufficient to perform adequate surgery in all cases, whereas there is a clear correlation between a more complete radical cytoreduction and prognosis. An instrument that can detect tumor cells more accurately during

surgery would assist the surgeon in obtaining a true R0 resection and thereby improving the outcome of the HIPEC procedure. This would decrease recurrence rate and improve disease free survival.
A targeted optical-imaging agent that highlights the tumor and enables to see whether the tumor is still present or not would be a valuable addition to the commonly used MRI and/or PET/CT. Another advantage is that it could also be possible to detect metastasis in pelvic lymph nodes and distant metastases and so enable upstaging and preventing unnecessary aggressive surgery in these patients.

1.4 NIRF intra operative imaging

In recent years, significant progress is made in the development of optical imaging systems and optical contrast agents, which are more tumour specific. In collaboration with the Technical University of Munich, a real-time optical imaging system has been developed using video-rate concurrent multi-spectral near-infrared and visible light imaging at a molecular level.[2-5] This system is currently in clinical use. After intravenous injection of an optical contrast agent, an external light source with a defined wavelength is used to illuminate the subject. As light propagates through the tissue, it will excite both surface and subsurface localized optical contrast agents.[5, 6] Immediately after excitation, the contrast agent responds by releasing low-energy light of a longer wavelength, which can subsequently be detected by a highly sensitive charge-coupled device (CCD) camera.[6] Recently published data show that this optical imaging system is able to detect solid tumors non-invasively in in vivo cancer mouse models with high sensitivity (in the picomolar range) and specificity (figure 1).[7]

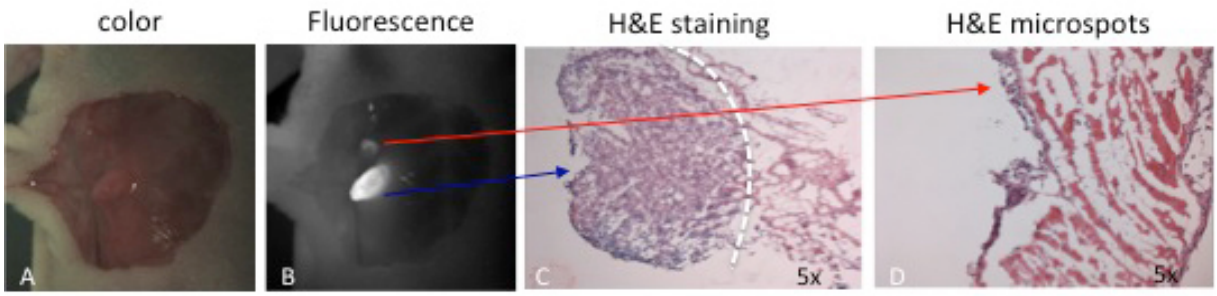

Figure 1: Breast cancer mouse model. A) after resecting 90% of the tumor tissue to intentionally leave behind 10% residual disease. B) fluorescence image show the 10% intentional left behind (blue arrow) and a non-intentional left behind microspot (red arrow). C) H&E staining showing tumor tissue and a rim of healthy muscle. D) H&E showing a small tumor deposit from the non intentionally, not visible for the naked eye microspot (red arrow).

The system was currently being used in pilot studies in various patient groups with cancer at the UMCG (ovarian cancer, cervical cancer, vulvar cancer, breast cancer and esophageal cancer). It must be emphasized that optical imaging by itself is not possible without the use of an optical contrast agent. In the

first pilot study in ovarian cancer study, the feasibility of the optical contrast agent folate-FITC (EC17) was investigated.

The intra-operative NIRF imaging camera in combination with this tumor-specific optical contrast agent is tested for its feasibility to detect residual disease in patients with ovarian cancer (METc study 2009.091). Based on the preliminary results of the pilot study in 10 patients (3 patients with ovarian cancer and 6 negative as defined after the surgical procedure with one patient with an inflammatory process), intra-operative imaging appears to be feasible and detects more tissue than visual inspection and tactile information alone (median of 11 spots by visual observation alone vs 25 spots with fluorescence, p=0.002).[8]

Figure 2 shows how this over-expression of FR in tumor cells (ovarian cancer) can be used to differentiate between malignant and healthy tissue in vivo.

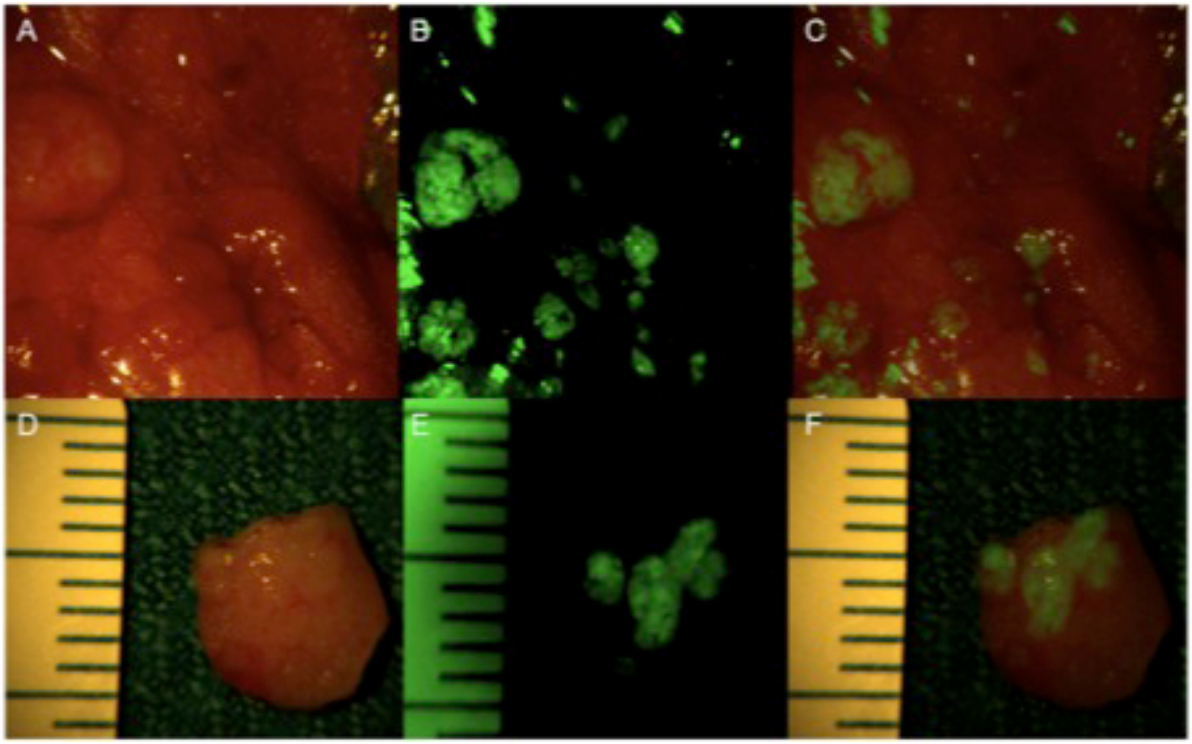

Figure 2: Intra-peritoneal metastases in human (A). Detection by using the folate-FITC optical contrast agent using fluorescence imaging (B). Signal overlaid on color image (C). Ex vivo biopsy of positive spot (D). Signal from Fr-FITC (E). Signal overlaid on color image (F)

Unfortunately the folate receptor has less expression in colorectal cancer patients in only 32% of the patients compared to 90% in ovarian cancer.[9]

Several biomarkers such as those involved in angiogenesis, including VEGF (Vascular Endothelial Grow Factor), have already been identified in colon cancer by our group in PC of colorectal origin. VEGF is a potent angiogenic growth factor, commonly involved in tumor-induced angiogenesis, with a putative therapeutical significance in the context of colon cancer.

Angiogenesis in colorectal carcinoma has been extensively studied. Several studies have determined that VEGF is up-regulated in colorectal cancer cells.[10-12] In our preliminary data, we have seen a significant upregulation of VEGF in cancer cells in PC of colorectal origin. This is also seen by Takahashi et al.[13] VEGF121 is freely soluble, whereas splice variants such as VEGF165, 189, 206 are mainly located in the extra cellular matrix, resulting in high concentrations in the tumor micro-environment. In conclusion, VEGF is highly upregulated in colorectal tumor tissue as well as in tumor tissue in PC of colorectal origin, and non-invasive assessment of VEGF-levels in the micro-environment of PC of colorectal cancer could potentially be used an imaging tool.

1.5 VEGF imaging

Systemic VEGF levels only consist of splice variants VEGF121 and a small proportion of VEGF165, whereas VEGF189, 206 and a large proportion of VEGF165 are mainly located in the extra cellular matrix, resulting in high concentrations in the tumor micro-environment. In 2002 Collingridge et al reported development of a radiolabeled antibody against VEGF, [124I]-SHPP-VG76e.[14] They found a high tumor to background contrast in a human fibrosarcoma xenograft 24 hours after injection. Another antibody against VEGF (HuMV833) was tested in an EORTC phase I study, and PET scanning with 124I-HuMV833 was used to measure distribution and clearance of the antibody. Great variation in distribution and clearance between patients and between different lesions within patients was found.
At the University Medical Center Groningen, non-invasive in vivo VEGF imaging with radiolabeled bevacizumab has been developed. Bevacizumab, which binds to all VEGF splice variants, can be labeled with the single γ-emitting isotope Indium-111 (111In) and with the PET isotope Zirconium-89 (89Zr) while preserving VEGF binding properties. In a human ovarian tumor xenograft, PET imaging 24 hours after injection of 89Zr-bevacizumab showed high uptake in well perfused organs and in the tumor, with an increase in tumor to background ratio in time, resulting in clear tumor localization after 72 hours.[15] Ranibizumab, a Fab-fragment directed at all VEGF-A splice variants, has also successfully been labeled with 111In, 89Zr and 18F at our institution. The shorter half- life of Fab-fragments compared to antibodies allows earlier imaging after injection. However, absolute tumor uptake is lower for Fab-fragments compared to intact antibodies.[16] In patients with melanoma, successful studies have been carried out at the University Medical Centre Groningen with 111In-bevacizumab SPECT. Preliminary results show that tumors can be visualized with variation in uptake between and within lesions in individual patients.[16] In addition, lesions < 1 cm were detectable using 111In-bevacizumab SPECT. Compared to SPECT imaging, PET imaging allows even more accurate attenuation and scatter correction, resulting in higher resolution, and uptake that can be better quantified.

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Recently Chang et al showed the feasibility of optical imaging of VEGF in a tumor bearing animal model.[17] VEGF was targeted similar to our strategy, however conjugated with a fluorescent dye, which is not suitable for early clinical use. In a CRC peritoneal carcinomatosis animal tumor model the in vivo intraoperative fluorescence imaging was performed with a clinical prototype multispectral normalized fluorescence imaging system. With this strategy, VEGF levels were successfully imaged quantitatively in tumors and showed concordance with results from VEGF levels measured in tumor tissue. These findings illustrate the feasibility of fluorescent optical imaging of VEGF in tumors. This is of great interest, as this relatively inexpensive and non-radioactive way of molecular imaging may be particularly suitable for PC detection during surgery. Recently published data demonstrate excellent tumor detection using NIR fluorescence labeled antibodies targeting VEGF in an animal model.[18]
In addition, at this moment a clinical trial in breast cancer patients (NL37479.042.11) is evaluating the use of bevacizumab-IRDye800CW for intraoperative-guided surgery. In the first thirteen patients the tumour could be visualised by bevacizumab-IRDye800CW. Also three patients with rectum carcinoma were included the RAPIDO trial (NL36315.042.11) and showed in situ fluorescent signals by endoscopy.
In addition, no toxicity of the tracer bevacizumab-IRDye800CW was observed and therefore declared safe.

1.5.1 Pre-clinical molecular imaging of VEGF

Bevacizumab (Avastin®, Roche) is a humanized antibody with anti-neoangiogenic properties and is used in the clinic extensively. It neutralizes all isoforms of VEGF-A. For imaging purposes in preclinical experiments, bevacizumab has been labeled with SPECT and PET isotopes and optical fluorophores.
In a human ovarian tumor xenograft, PET imaging 24 hours after injection of ^{89}Zr-bevacizumab showed high uptake in well perfused organs and in the tumor, with an increase in tumor to background ratio in time, resulting in clear tumor localization after 72 and 168 hours.[15]
At the University Medical Center Groningen, antibody-based tumor detection for optical imaging using preclinical in vivo mouse models was used. Bevacizumab was labeled with IRDye 800CW. Tumour uptake of the fluorescent tracers and zirconium-89 (^{89}Zr) labeled radioactive counterparts for PET imaging, was determined in human-xenograft-bearing athymic mice during one week after tracer injection, followed by ex vivo biodistribution and pathological examination. Intra-operative imaging of fluorescent VEGF-positive tumor lesions was performed in subcutaneous tumors and in intraperitoneal tumour dissemination models.[18] Tumour-to-background ratios, with fluorescent imaging, were 1.93 ± 0.40 for bevacizumab-IRDye800CW three days after tracer administration. Intra-operative imaging even detected tumor lesions at the sub-millimeter level in intraperitoneally-disseminated tumor models. These results were supported by histology, immunohistochemistry and fluorescence microscopy analyses.

1.5.2 Clinical Molecular Imaging of VEGF

Successful clinical imaging of VEGF with SPECT and PET labeled bevacizumab as tracer has already been demonstrated in patients with stage III/IV melanoma and renal cell cancer in feasibility studies at the University Medical Center Groningen. In one study, ^{111}In-bevacizumab was used to visualize melanomas in 9 patients.[19] FDG-PET and CT detected both in total 12 nodal lesions, which were all visualized by ^{111}In-bevacizumab. At baseline, ^{111}In-bevacizumab tumor uptake varied 3-fold between and 1.6±0.1-fold within patients. The ^{111}In-bevacizumab tumor uptake positively correlated with the VEGF-A expression in the resected tumors. At the University Medical Center Nijmegen, ^{111}In-bevacizumab was used to depict renal cell carcinoma and response to neoadjuvant therapy[20]. This study showed preferential accumulation of ^{111}In-bevacizumab and correlation with VEGF-A levels. Treatment of the patients resulted in a significant decrease of uptake of ^{111}In-bevacizumab.

In an ongoing study (NCT00991978) in patients with early stage breast cancer, uptake of ^{89}Zr-bevazicumab in primary breast tumor was assessed in 23 patients. Imaging was performed 3 to 4 days after tracer administration. In 25 tumors out of 26 tumors, the tumor could be visualized. Furthermore, up until now more than 120 patients have been included in clinical studies with ^{89}Zr-bevacizumab. No bevacizumab-related adverse events were seen in any of these patients.

These results show that tumors can be visualized with variation in uptake between and within lesions and between patients.

1.6 Development and manufacturing of the VEGF-targeting optical fluorescent tracer bevacizumab-IRDye800CW for use in clinical studies

As indicated above, radio-isotope labeled bevacizumab could be a good candidate tracer to identify and characterize lesions and to give information of whole body drug distribution. However, as a radiation dose is administered, the patient is exposed to systemic radiation. Moreover, nuclear imaging requires an advanced and complex infrastructure in terms of production, transportation and safety aspects and is therefore very expensive. Fluorescent labeled bevacizumab does not use radioactive components or ionizing radiation and could thus be used more frequently. Moreover, the fluorescent labeled bevacizumab is easier produced at lower costs than radio-isotope labeled bevacizumab and is very stable over time; a shelf life of several months has been recorded in the University Medical Center Groningen.

The near-infrared organic fluorophore IRDye800CW (800CW-NHS ester), developed by LI-COR Biosciences (Lincoln, NE, USA), can be used for near-infrared imaging in tissue which has been shown in pre-clinical experiments to be safe and non-toxic.[21] See Active Substance Master file for IRDye800CW documentation. Labeling of bevacizumab (and other tumor targeting agents) to IRDye 800CW has been

performed at the UMC Groningen through the NHS-ester present in the compound. Bevacizumab was labeled with IRDye800CW in a molar ratio of 1 to 4. IRDye 800CW was bound to lysine amino acids in the protein followed by purification, formulation and final sterilization. The conjugation resulted in approximately 3.5 fluorophores per antibody molecule. Bevacizumab-IRDye800CW was produced at a clinical grade at the biotech unit of the UMC Groningen, meeting all GMP-requirements. Quality control was conducted assuring identity, quality, purity and biological activity of the tracer. Detailed information about the tracer bevacizumab-IRDye 800CW can be found in the Investigational Medicinal Product Dossier.

1.7 Application of optical imaging techniques

Many clinical optical imaging systems able to measure fluorescent signals in human subjects are in various stages of development. At our institutions, three high-end clinical optical imaging systems capable of fluorescence imaging in the near-infrared range are available. For screening/diagnostic applications a hand-held Multispectral Opto-acoustic Tomography (MSOT) system (Technical University Munich, Munich, Germany), a Multispectral Fluorescence Reflectance Imaging (MFRI) camera system (Technical University Munich, Munich, Germany) designed for intra-operative applications and a multispectral NIR fluorescence laparoscopy system, which can be attached to the intraoperative system, are available in the UMC Groningen. The MFRI camera system in combination with bevacizumab-IRDye800CW was able to visualise clearly all breast tumours in the first thirteen patients of the breast cancer trial (NCT00991978) and three rectum carcinoma patient, included the RAPIDO trial (NL36315.042.11), without showing any adverse events.

1.7.1 Multispectral Fluorescence Reflectance Imaging (MFRI)

The use of NIR fluorescence optical imaging has a range of advantages (e.g. compared to radioactive imaging). Most prominent among these are the safety related aspects, ease of operation, high speed, high resolution (as low as 10 μm), relatively low costs and the use of non-ionizing radiation.[22] Since March 2009, a clinical prototype Multispectral Fluorescence Reflectance Imaging (MFRI) intra-operative camera system, developed together with the Technical University of Munich, to which the NIR fluorescence laparoscope scope is attached, is being used in the UMC Groningen in various applications such as sentinel lymph node mapping in breast cancer patients using indocyanine green and tumor-targeted imaging in ovarian cancer patients using Folate-FITC, targeting the folate receptor.[3, 8] In addition, at this moment a clinical trial in breast cancer patients (NL37479.042.11) and the RAPIDO trial (NL36315.042.11), are evaluating the use of bevacizumab-IRDye800CW for intraoperative-guided surgery and endoscopic evaluation of response after chemo and radiotherapy. These clinical studies were approved by the ethics

committee of the University Medical Center Groningen (CCMO NL26981.042.09, NL26982.042.09, NL26980.042.09 and NL37479.042.11). Recently, the first-in human proof-of-principle results from these studies have been published.[8] In the first thirteen patients of the breast cancer trial (NL37479.042.11) the tumour could be visualised by bevacizumab-IRDye800CW. In addition, no toxicity of the tracer bevacizumab-IRDye800CW was observed.

1.8 Conclusion: motivation for the feasibility of NIR fluorescence imaging in Peritoneal Carcinomatosis

Preliminary results from an ongoing trial in melanoma patients using 111In-bevacizumab SPECT imaging shows high VEGF levels in tumor lesions. All known lesions could be detected, even lesions < 1 cm. Lesions < 1 cm should be easily revealed with bevacizumab-IRDye800CW in high-resolution intraoperative fluorescence imaging. In regards to colorectal cancer, normal tissue has a significantly lower VEGF excretion than tissue containing colorectal cancer cells. Bevacizumab-IRDye800CW uptake can be quantified ex vivo, which will lead to both a high sensitivity and specificity of this technique, Better intraoperative imaging may reduce the number of patients undergoing unnecessary surgery. Recently we have shown that this concept is feasible in using a tumor-specific folate-receptor alpha targeted probe in patients with ovarian cancer[7] with a resolution of less than 1 mm.

In the present study, we aim to perform a feasibility study to prove that bevacizumab-IRDye800CW with intraoperative fluorescence scanning can indeed detect cancer lesions (probably <1 mm) in PC of colorectal origin. Data from the present study may be used to design further studies with regard to intraoperative fluorescent detection of cancer lesions in PC in a sufficiently powered final multicenter diagnostic accuracy study, and can potentially support further development of other fluorescent optical imaging with different targets in solid tumors.

2. Objectives

2.1 Primary objectives

Determining the feasibility in terms of sensitivity and specificity of intraoperative fluorescence imaging detection of peritoneal carcinomatosis of colorectal origin using the VEGF-targeting optical agent bevacizumab-IRDye800CW as confirmed by ex vivo standard H&E staining.

2.1.1 Research aims to assess primary objective

Determination of sensitivity ands specificity data of the sampled (non-) fluorescent tissue acquired intraoperatively compared to standard histopathological examination for the presence of tumor tissue. (10 samples per patient).

Localization and (semi) quantification of a fluorescent signal of tumor tissue and surrounding tissue after post-processing of the in vivo required data.

In vivo NIR fluorescence quantification vs. *ex vivo* VEGF levels in biopsies and additional *ex vivo* analyses (using immunohistochemistry (IHC).

Evaluation of safety aspects

To obtain information on safety aspects of the tracer, side effects, adverse events (AE), serious adverse events (SAE) and suspected unexpected serious adverse reactions (SUSAR).

2.2 Secondary objectives

Improving the detection rate of peritoneal carcinomatosa using MFR imaging, for purposes of better staging by calculating the peritoneal carcinomatosis index (PCI) based on fluorescence detection

2.1.2 Research aims to assess secondary objective

Determining the feasibility of intra-operative detection of peritoneal carcinomatosis of colorectal origin with intraoperative fluorescence imaging using the VEGF-targeting optical agent bevacizumab-IRDye800CW. To demonstrate if the detection rate of tumor tissue will be increased, the peritoneal cancer index will be determined by visual and tactile inspection alone and with the fluorescent images.

3. Study design

The Hi-Light study is a, non-blinded, prospective, single center feasibility study. Bevacizumab-IRDye800CW will be administered to a total of ten (10) patients with proven peritoneal carcinomatosis from colorectal origin. Administration of bevacizumab-IRDye800CW will take place in the UMC Groningen. For all patients injected with the tracer, intra operative multispectral Fluorescence Reflectance Imaging (MFRI) will be performed. No additional blood samples are collected in this side study. The design of this study warrants maximal data collection, while risks and burden for patients are minimised. Also, the framework of this study can be used for evaluation of other, new developed (fluorescent) tracers.

The first stage of the study will be evaluated after five (5) patients. The study will be suspended if no accumulation of Bevacizumab-IRDye800CW in the tumour tissue can be demonstrated in these patients (5/5). The study will also be suspended immediately if any serious adverse event related to the administration of the tracer occurs in any of the patients.
It is known from studies using radioactive bevacizumab that the uptake of the tracer increases in time.[19] From preclinical studies, we also know that most of the uptake is already present at day 1 after administration.[18] In the present study, Multispectral Fluorescence Reflectance Imaging will be performed at day 2 after administration of bevacizumab-IRDye800CW.

Summary of patient related study procedures
The study procedures are described extensively in section 6.3.
Visit 1: screening visit
Screening of patients, informed consent, collection of patient characteristics and physical examination.
Visit 2: tracer administration
Two days prior to surgery. Administration of tracer Bevacizumab-IRDye800CW and safety monitoring (up till 1 hour after tracer administration).
Visit 3: operation date
The patients will be admitted to the hospital one or two days prior to surgery. During surgery, the extent of tumor tissue will be described as by using the peritoneal carcinomatosis index (PCI), without the intraoperative camera system and also by using the camera system (PCI as described by Sugarbaker et al.[23])

Summary of biopsy specimen related procedures
In each patient a total of 10 samples (5 fluorescent samples and 5 non-fluorescent samples) with a maximum size of 5 mm will be taken for additional analyses either by standard histopathological examination (H&E) or IHC. The samples will be divided in half and stored either in formalin or snap-frozen. The biopsies will be taken from the resected tissue and will be sampled out of the patient. This will therefore not prolong the operation time.

4.Study population

4.1 Population (base)

Patients admitted to the UMCG to undergo cytoreductive surgery for peritoneal carcinomatosis of colorectal cancer are eligible for this study. Yearly, around 25-30 surgical cytoreductive procedures with

HIPEC for colorectal cancer are carried out by the surgical oncologists at the UMCG. Taking into consideration that 80% of all patients can be recruited is based on the experiences in disseminated ovarian cancer, it is likely that the proposed number of 10 patients for this phase I technical feasibility study is feasible within a time-frame of 35 weeks. The characteristics of the study population are patients above the age of 21 with proven colorectal cancer with peritoneal carcinomatosis with an indication to undergo cytoreductive surgery.

4.2 Inclusion criteria

Age ≥ 18 years.

Patients with histopathological proven peritoneal carcinomatosis from colorectal origin who are scheduled to undergo the HIPEC procedure

Patient is considered to be mentally and physically fit for the HIPEC procedure as judged by the responsible physician

WHO performance score 0-2

Signed written informed consent.

4.3 Exclusion criteria

Concomitant malignancies, except for adequately treated basocellular carcinoma of the skin or in situ carcinoma of the cervix uteri. Subjects with prior malignancies must be disease-free for at least 5 years.

Distance metastasis (liver / lungs)

Medical or psychiatric conditions that compromise the patient's ability to give informed consent.

Concurrent uncontrolled medical conditions.

Pregnancy or breast feeding.

Clinically significant (i.e. active) cardiac disease (e.g. congestive heart failure, symptomatic coronary artery disease and cardiac dysrhythmia, e.g. atrial fibrillation, even if controlled with medication) or myocardial infarction within the past 12 months.

4.4 Sample size calculation

The current study design is a feasibility pilot study in which a total number of ten (10) patients is considered useful for evaluation of intraoperative imaging using bevacizumab-IRDye800CW. No calculation on the number of patients to include can be made because of the design of the study and the lack of reference data. If the results of the first five (5) patients are promising, defined as a positive signal detection in 3/5 patients as determined by a blinded surgeon, the intermediate result of the pilot study is considered

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successful and can be carried on. The final result is regarded successful if 7/10 patients do have targeting of tumor tissue. Based on the comparison of the samples taken from fluorescent and non-fluorescent tissue and comparison with standard histopathological examination (H&E), a reliable power analysis for a multicenter diagnostic accuracy test can be performed in a follow-up phase II study.

5. Investigational Medicinal Product

The Clinical Trial Application presents information related to the monoclonal antibody bevacizumab labelled with the fluorescent dye IRDye800CW as injection. The injection vial contains 5.0 mg bevacizumab-IRDye800CW in 0.9% NaCl. Patients will receive a single dose bevacizumab-IRDye800CW of 4.5 mg by intravenous administration, according to the IMPD. Bevacizumab (Avastin®, Roche) is a recombinant, high affinity, humanized IgG1 monoclonal antibody with specific affinity for VEGF. The infrared dye IRDye800CW (LI-COR Biosciences, Lincoln, NE, USA) is a fluorescent dye applicable for clinical use, produced by REGIS technologies. Conjugation of the fluorescent dye to bevacizumab, purification and formulation will be performed at the department of hospital and clinical pharmacy of the UMC Groningen. The new tracer bevacizumab-IRDye 800CW has been evaluated preclinical in tumor bearing nude mice and an extended single microdose toxicity study has been performed by NOTOX, which did not show toxicity (more information can be found in the IMPD).
Patients will receive a single dose of 4.5 mg microdosing of bevacizumab-IRDye800CW, compared to a therapeutic dose of 5-10 mg/kg every two weeks. Recently, 26 renal cancer patients underwent repeated 4.5 mg ^{89}Zr-bevacizumab administrations and imaging at baseline, 4 weeks and 6 weeks at the UMCG (NCT00831857). One patient reported nausea, redness of the face and cold extremities for 24 hours after the third tracer injection but continued bevacizumab treatment (10 mg/kg) without adverse events. No toxicity was observed in the first thirteen breast cancer (NL37479.042.11) and three rectum carcinoma patients (NL36315.042.11), using bevacizumab-IRDye800CW. Based on these we do not expect toxicity of the second micro-dose administration of bevacizumab-IRDye800CW. Off course, patients will be extensively observed during the study to notify any adverse events.
More detailed information about the investigational medicinal product is described in the IMPD of bevacizumab-IRDye800CW.

6. Methods

6.1 Study parameters and reference standards

Determining the feasibility of pre-operative detection of peritoneal carcinomatosis of colorectal origin by intraoperative fluorescence imaging using the VEGF-targeting optical agent bevacizumab-IRDye800CW as

confirmed by ex vivo standard histopathological and immunohistochemical analyses for the presence of tumour cells and VEGF-A expression in excised tumor tissue en fluorescence microscopy for the bevacizumab-IRDye800CW tracer.

6.1.1 Primary study parameters and reference standards

Parameters

Macroscopic fluorescent signal levels observed by intraoperative Multispectral Fluorescence Reflectance Imaging and tracer distribution in biopsy specimens (semi-quantitative) compared to the presence or absence of tumour tissue as determined by standard H&E histopathological analyses.

Microscopic fluorescent signal levels observed and distribution in biopsy specimens (semi-quantitative).

Adverse events (AE), serious adverse events (SAE), and suspected unexpected serious adverse reactions (SUSARs).

Reference standards

Histologically ascertained tissue types (qualitative):

Normal peritoneal tissue

Tumour tissue and specific associated tissue types (tumour, stroma)

Tissue VEGF levels (immunohistochemistry) and other related staining.

To correlate the fluorescent signal assessed by NIR fluorescence endoscopy with other biological and molecular parameters (IHC, DNA and RNA analyses, protein analyses) and the fluorescent signal assessed in the *ex vivo* biopsy specimens.

6.1.2 Other study parameters

Patient characteristics (age, history, outcome of procedures)

Tumour characteristics (size, stage, location, imaging characteristics, microsatellite-instability status, KRAS / BRAF status, p53 mutations etc.)

Histopathologic examinations related to VEGF.

6.2 Randomization, blinding and treatment allocation

Video's and still images of relevant areas will be evaluated by a engineer from the Technical University of Munich blinded for clinical information and location of tumor tissue.

6.3 Study procedures

A summary of the study procedures can be found in section 3.

	Screening Visit	Tracer injection	Operation date
Patients with peritoneal carcinomatosis from colorectal origin	At Inclusion	2 days prior to surgery	Intraoperative Multispectral Fluorescence Reflectance Imaging (MFRI)

6.3.1 General study procedures

General clinical practice will have priority over study procedures at all times.

Patients with peritoneal carcinomatosis of colorectal origin, without metastases to liver and or lungs, who are scheduled for cytoreductive surgery and a HIPEC procedure, will be consented for this study in the outpatient clinic. The patients are asked for participation and informed consent for the proposed study if they apply to the inclusion criteria for this pilot study (according to paragraph 4.2 and 4.3). Copies of the proposed study protocol are available at the outpatient clinic.

Histology confirming diagnosis is standard in the pre-operative work-up of all patients undergoing the HIPEC procedure. After informed consent is obtained and properly documented, the CT- thorax, abdomen, and possibly PET-scan will be re evaluated to be sure that no metastasis to liver and or lung are present. Eligibility is assessed by checking in- and exclusion criteria collecting necessary data on history, physical and laboratory examination from the medical file of the patient. If the patient is eligible to participate in the study, a sheet containing information about the study procedures of the Hi-Light study is handed to the patient. The patient has seven (7) days after he/she receives the information to decide whether he/she wants to participate in the study. Potential eligible patients are identified by the study physician and discussed in the multidisciplinary HIPEC meeting at the UMCG.

As soon as the patient is included, a personal subject number is assigned to all participating patients. Furthermore, a code list is produced for data management after the cytoreductive surgery and HIPEC procedure and stored at the desk of the principal investigator. Finally, the operative procedure is planned.

Visit 1: Screening visit

Screening of patients, informed consent, collection of patient characteristics, physical examination

When patients decide to participate in the Hi-Light study, written informed consent is asked (informed consent sheets for UMC Groningen). Appointments for the administration of the tracer administration can

be made when the operation date is know. Patient data are collected. The following information will be recorded:

Demographic data, personal and family medical history (colon cancer specific).

Available imaging and pathology data.

General physical examination (including at least weight, height, vital signs (blood pressure, pulse, temperature), examination of lungs, heart, abdomen, skin (moles, scars) and lymph nodes).

Visit 2: Tracer administration

Tracer administration and safety monitoring will occur two days prior to surgery

At the day of administration of the tracer, the patient is asked for signs/symptoms present after the screening visit (baseline findings). Before administration of the tracer, vital signs will be measured. An intravenous line will be installed. A single dose of 4.5 mg of Bevacizumab-IRDye800CW will be administered intravenously to the patient at the ward of the Division of Surgical Oncology, K4VA at the UMCG. The infusion line will be flushed with a saline solution afterwards. After tracer administration, blood pressure, pulse and temperature will be measured. The patient will be observed for 60 minutes following tracer injection. Vital signs will be measured after 15, 30, 45 and 60 minutes. During safety monitoring, a crash car with necessary equipment is available in case of an adverse reaction. After the observational period of 1 hour, the intravenous line will be removed and the patient will be discharged.

Visit 3: HIPEC procedure

The operative procedure two days after i.v. injection, is executed as normal. After laparotomy the surgeon will estimate the peritoneal carcinomatosis index (PCI) by visual and tactile observation alone. Next, the same surgeon will score the PCI by support of the NIRF imaging system. Subsequently, the actual cytoreductive procedure will be carried out first by visual observation alone. When the surgeon decides that on the basis of his visual and tactile observation all tumor tissue is removed, the abdomen will be inspected by fluorescent intraoperative imaging which is estimated to take 10 minutes. After the cytoreduction the presence of tumor-bound Bevacizumab-IRDye800CW is assessed by placing the optical imaging device 20 cm above the region of interest (operative field) and therefore will not intervene with the actual procedure. Additionally, optical images will be taken from the abdominal cavity for the possibility to detect residual disease. In case of fluorescence, additional tissue will be removed if technically safe as judged by the attending experienced HIPEC surgeon, and analyzed separately by the pathologist. This imaging procedure may result in additional operating time up to 45 minutes. Also normal photographs and videos of the operative field are taken for the sake of comparison. Imaging data (by date, time and subject identification code) are stored temporarily on a stand-alone computer. This is the end of the imaging

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procedure and the surgery can be continued as normal. While all sampling procedures of the resected specimen will be done off-table.
After the cytoreduction and the additional imaging the procedure will be continued as normal. This means flushing the abdomen using Mitomycin C in 41 degrees Celsius. After the 90 minutes of perfusion the remaining procedure will be performed. After the surgical procedure, the patients will be monitored on the intensive care unit as usual after a HIPEC procedure.

The excised tissue fragment(s) will be visualized outside the patient with the optical imaging device for the presence of tumor-bound Bevacizumab-IRDye800CW, taking samples of both fluorescent (five) and non-fluorescent tissue (five) of peritoneum or non-fluorescent suspicious tumor spots. These samples will be used to evaluate sensitivity and specificity.
Thereafter, the excised tissue will be send fresh to the pathology department for histopathological analysis (standard procedure). Also, all fragments of tumorload excised during the cytoredution will be assessed for the presence of tumor-bound Bevacizumab-IRDye800CW by fluoresence microscopy. Characteristics of the tumor will be recorded and stored in an anonymous format by the principal investigator.

6.3.2 Specimen related study procedures

Fresh biopsy specimen procedures

The biopsies collected in the Hi-Light study will be used for study-procedures only and will therefore not be included in any routine histopathological examinations. The origin of the samples will be recorded digitally and coded during the procedure.

1. Paraffin embedding

The fresh biopsy specimens will be fixed in formalin in the dark and processed further to paraffin embedding afterwards.

2. Freezing

Alongside to fresh biopsy specimens collected for paraffin embedding, samples will be collected for analyses specifically performed on frozen material. Immediately after the biopsies are taken, the specimens will be frozen in -80 degrees Celsius in the OR.

Fixed biopsy specimen procedures

Remark: The pathological study procedures and examinations that will be performed on the study material will be encrypted and performed in batches after all patient procedures have been completed for an interim analyses of five (5) patients.

1. Paraffin embedded specimen procedures

The paraffin embedded specimens will be cut in micrometer thin sections and mounted on glass slides to be imaged for localisation and of the fluorescent signal and assessment of heterogeneity, using the Odyssey Infrared Imaging System (LI-COR Biosciences, GmbH). Back-to-back hematoxylin and eosin (HE) stained, IHC stained (for VEGF, CD34 for micro vessel density (MVD), SMA for vascular maturation, HIF-1alpha for hypoxia, Ki-67 for proliferation and other relevant markers) and unstained slides (for fluorescence) will be investigated by (fluorescence) microscopy for semi-quantification and assessment of the (sub)cellular location of the tracer.

2. Frozen specimen procedures

DNA and RNA analyses will be performed using the Affymetrix® platform to get insight in upregulated pathways in areas with high and low fluorescent signals.

6.4 Withdrawal of individual subjects

Subjects can leave the study at any time for any reason if they wish to do so without any consequences. The investigator records the reason of withdrawal if possible. The investigator can decide to withdraw a subject from the study for urgent medical reasons, withdrawal should be considered in case of a serious adverse event.

6.4.1 Specific criteria for withdrawal (if applicable)

Not applicable

6.5 Replacement of individual subjects after withdrawal

A total of ten (10) patients for colorectal cancer will be recruited in this feasibility pilot study. If a patient withdraws from the study before the procedure, a new patient will be recruited until the total number of ten (10) patients is reached. If the results of the first five (5) patients are successful (defined as a positive signal in tumor tissue in 3/5 patients confirmed by ex vivo immunohistochemistry or fluorescence microscopy of excised specimen, the intermediate results of the study are considered successful and will carried out completely until the inclusion of 10 patients.

6.6 Follow-up of subjects withdrawn from treatment

After administration of the tracer, (serious) adverse events that occur in subjects that are withdrawn from the study procedure will still be recorded if possible.

6.7 Termination of the study

6.7.1 Termination based on safety aspects

The research team with all participating study investigators involved will discuss safety aspects during the study procedures. Results will be reported immediately in case of any SUSAR. After the first 3 patients the (serious) adverse events ((S)AE) will be reported to the METc. The study will be terminated in case a suspected unexpected serious adverse reaction (SUSAR) occurs in any of the patients until 2 weeks after administration of bevacizumab-800CW, as will be discussed with the METc.

6.7.2 Termination based on Bevacizumab-IRDye800CW accumulation

The study will be terminated if after an interim analysis of the first 5 evaluable patients no uptake of Bevacizumab-IRDye800CW in tumour tissue can be shown by fluorescence imaging in 2/5 patients by any of the available technologies. See paragraph 4.4 for statistical considerations.

6.7.3 Termination based on other aspects.

The study will be suspended based on urgent medical or ethical considerations as decided by the principal investigators. In case of termination of the study, the institutions, regulatory authorities, CCMO and the METc of the study center will be informed.

7. Safety monitoring

7.1 Section 10 WMO event

In accordance to section 10, subsection 1, of the WMO, the investigator will inform the subjects and the reviewing accredited METc if anything occurs, on the basis of which it appears that the disadvantages of participation may be significantly greater than was foreseen in the research proposal. The study will be suspended pending further review by the accredited METc, except insofar as suspension would jeopardize the subjects' health. The investigator will take care that all subjects are kept informed.

7.2 Adverse and serious adverse events

Adverse events are defined as any undesirable experience occurring to a subject during a clinical trial, whether or not considered related to the investigational drug. All adverse events reported spontaneously by the subject or observed by the investigator or his staff will be recorded.

A serious adverse event is any untoward medical occurrence or effect that at any dose:

results in death;

is life threatening (at the time of the event);

requires hospitalization or prolongation of existing inpatients' hospitalization;

results in persistent or significant disability or incapacity;

is a congenital anomaly or birth defect;

is a new event of the trial likely to affect the safety of the subjects, such as an unexpected outcome of an adverse reaction, lack of efficacy of an IMP used for the treatment of a life threatening disease, major safety finding from a newly completed animal study, etc.

All SAEs will be reported through the web portal ToetsingOnline to the accredited METC that approved the protocol, within 15 days after the sponsor has first knowledge of the serious adverse reactions. SAEs will be also reported to Roche.
SAEs that result in death or are life threatening will be reported expeditiously. The expedited reporting will occur not later than 7 days after the responsible investigator has first knowledge of the adverse reaction. This is for a preliminary report with another 8 days for completion of the report.

7.2.1 Suspected unexpected serious adverse reactions (SUSAR)

Adverse reactions are all untoward and unintended responses to an investigational product related to any dose administered. Unexpected adverse reactions are adverse reactions, of which the nature, or severity, is not consistent with the applicable product information (e.g. Investigator's Brochure for an unapproved IMP or Summary of Product Characteristics (SPC) for an authorised medicinal product). The sponsor will report expeditiously the following SUSARs through the web portal ToetsingOnline to the METC:

SUSARs that have arisen in the clinical trial that was assessed by the METC;

SUSARs that have arisen in other clinical trials of the same sponsor and with the same medicinal product, and that could have consequences for the safety of the subjects involved in the clinical trial that was assessed by the METC.

The remaining SUSARs are recorded in an overview list (line-listing) that will be submitted once every half year to the METC. This line listing provides an overview of all SUSARs from the study medicine, accompanied by a brief report highlighting the main points of concern. The expedited reporting of SUSARs through the web portal ToetsingOnline is sufficient as notification to the competent authority.
The expedited reporting will occur not later than 15 days after the sponsor has first knowledge of the adverse reactions. For fatal or life threatening cases the term will be maximal 7 days for a preliminary report with another 8 days for completion of the report.

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7.2.2 Annual safety report

In addition to the expedited reporting of SUSARs, the sponsor will submit, once a year throughout the clinical trial, a safety report to the accredited METC, competent authority, Medicine Evaluation Board and competent authorities of the concerned Member States.
This safety report consists of:

a list of all suspected (unexpected or expected) serious adverse reactions, along with an aggregated summary table of all reported serious adverse reactions, ordered by organ system, per study.

a report concerning the safety of the subjects.

a complete safety analysis and an evaluation of the balance between the efficacy and the harmfulness of the investigational medicinal product under investigation).

7.3 Follow-up of adverse events

All adverse events will be followed until they have abated, or until a stable situation has been reached. Depending on the event, follow up may require additional tests or medical procedures as indicated, and/or referral to the general physician or a medical specialist.

7.4 Data Safety Monitoring Board (DSMB)

A Data Safety Monitoring Board will be installed consisting of prof JE Tulleken (intensive care), prof J. Pruim (nuclear medicine) and dr. RFE Wolff (radiologist).

7.5 Safety and expected side-effects

Bevacizumab-IRDye800CW safety aspects
Based on animal toxicity data, preclinical tracer evaluation data provided by NOTOX (see the IMPD), and experience in thirteen (13) patients in clinical trial evaluating the uptake of *Bevacizumab-IRDye800CW* in breast cancer patients (NL37479.042.11) no side-effects are anticipated. Adverse Events may be expected after administration of a low dose of bevacizumab. Hypersensitivity reactions to bevacizumab can occur within a short term after administration (up until 1 hour). Also, hypertension can occur after bevacizumab administration. However, the risk is considered minimal due to the low tracer dose used (micro-dose) in this study and is temporal without clinical consequences. Patients will receive a single dose of 4.5 mg micro-dosing of bevacizumab-IRDye800CW, with afterwards one hour of observation. Recently, 26 renal cancer patients underwent repeated ^{89}Zr-bevacizumab administrations and imaging at baseline, 4 weeks and 6 weeks at the UMCG (NCT00831857). One patient reported nausea, redness of the face and cold

extremities for 24 hours after the third tracer injection but continued bevacizumab treatment (10 mg/kg) without adverse events. Safety evaluation will be performed after each patient. Is is anticipated that any adverse event will not preclude the HIPEC procedure itself based on the experience with microdosing injection of bevaciuzmab-800CW sofar.

8. STATISTICAL ANALYSIS

8.1 Descriptive statistics

Descriptive statistics will include measures of distribution: (geometric) means with standard deviation; medians with range; frequencies. Continuous variables will be inspected for normal distribution by histograms, and if non-normally distributed, attempts will be made to transform the data to obtain a normal distribution.

8.1.1 Patient and tumor characteristics

Patient: age, skin type, BMI, signs/symptoms.

Tumour: stage, size, location, tumour characteristics, grade, histopathological VEGF-A expression.

8.1.2 Intraoperative Multispectral Fluorescence Reflectance Imaging (MFRI)

Semi-quantification of signal intensity of different areas (high and low signal intensity within the tumor, tumour margins, surrounding epithelium) (mean, sd area of tumour and surrounding tissue for all samples together (intra- and inter variability)).

Biopsies taken during HIPEC procedure (number per individual, fluorescence yes/no, %).

Imaging procedure time.

Qualitative visibility of a fluorescent signal in tumour and surrounding tissue (yes/no, %) on recorded location during the HIPEC procedure

8.1.3 Ex vivo biopsy specimens

Immunohistochemical determination of VEGF status of the biopsies (n, - / + / ++).

Other immunohistochemical stainings (positive/negative, %).

Histopathologic examination (tumor type, microsatellite-instability status, KRAS / BRAF status, etc.).

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8.2 Univariate analysis

We will calculate the sensitivity and specificity by asking a pathologist blinded for the imaging result to determine tumour positive and tumour negative (non-) fluorescent specimen by standard H/E. This will be correlated with the fluorescent signals. The sensitivity and specificity can be calculated.

	HE +	HE -
Fluorescence +	TP	FP
Fluorescence -	FN	TN

Sensitivity = True positive / (true positive + false negative)

Specificity = True negative / (true negative + false positive)

The difference between the PCI between the normal (visual and tactile inspection) and the PCI using fluorescence imaging will be analysed using a the nonparametric Wilcoxon t-test.

8.3 Multivariate analysis

By taking into account the size, grade, differentiation, p53 mutation and extension of metastasis as potential modifiers for the outcome this will be analysed using a multivariate analysis. (WANOVA)

8.4 Interim analysis

An interim analysis will be performed after the first five evaluable patients assessing the primary endpoint (Bevacizumab-IRDye800CW accumulation in the tumour). If no accumulation can be detected in 2 out of 5 patients by any of the available technologies, the study will be terminated (see also sections 4.4 and 6.7.2).

9. ETHICAL CONSIDERATIONS

9.1 Regulation statement

The study will be conducted according to the principles of the Declaration of Helsinki (Seoul 2008 amendment) and in accordance with the medical Research Involving Human Subjects Act (WMO) and other guidelines, regulations and Acts.
The protocol has been written and the study will be conducted according to the ICH Harmonized Tripartite Guideline for Good Clinical Practice. The protocol will be approved by the Local, Regional or National Ethics Committees.

9.2 Recruitment and consent

All patients will be informed about the aims of the study, the possible adverse events, the procedures and possible hazards to which they will be exposed before enrolment into the study. They will be informed as to

the strict confidentiality of their patient data. Their medical records will only be reviewed by authorised individuals other than their treating physician to check their eligibility for this study. Each patient will be given the opportunity to ask questions and will be informed about the right to withdraw from the study at any time without prejudice. See the patient information sheet and patient informed consent statement for the UMC Groningen.
Documented informed consent must be obtained for all patients included in the study before they are registered in the study. Patients must be given adequate opportunity to read the information and enquire about details of the study before consent is given.
The informed consent procedure takes place conform the ICH guidelines on Good Clinical Practice. This implies that the written informed consent form will be signed and personally dated by the patient or by the patient's legally acceptable representative. The informed consent statement will be signed and dated by the investigator afterwards and the patient will receive a copy.
The general physician of each patient will be informed about the enrolment of the patient to the study.

9.3 Benefits and risks assessment

For the participating patients, there is no diagnostic or treatment benefit related to the study.
Participation may possibly produce useful scientific data for the future.
Risk related to the investigational procedures are described in sections 5 and 7.5.

9.4 Compensation for injury

The sponsor/investigator has a liability insurance, which is in accordance with article 7, subsection 6 of the WMO.

The sponsor (also) has an insurance which is in accordance with the legal requirements in the Netherlands (Article 7 WMO and the Measure regarding Compulsory Insurance for Clinical Research in Humans of 23th June 2003). This insurance provides cover for damage to research subjects through injury or death caused by the study.
€ 450.000,-- (i.e. four hundred and fifty thousand Euro) for death or injury for each subject who participates in the Research;
€ 3.500.000,-- (i.e. three million five hundred thousand Euro) for death or injury for all subjects who participate in the Research;

€ 5.000.000,-- (i.e. five million Euro) for the total damage incurred by the organisation for all damage disclosed by scientific research for the Sponsor as 'verrichter' in the meaning of said Act in each year of insurance coverage.

The insurance applies to the damage that becomes apparent during the study or within 4 years after the end of the study.

9.5 Incentives

For each day of patient related study procedures, the subjects will receive compensation for travelling expenses (€ 0.19/km) and a ticket for free parking.

10. ADMINISTRATIVE ASPECTS AND PUBLICATION

10.1 Handling and storage of data and documents

Data will be handled confidential and anonymously. Because of information obtained during surgery and the definitive pathology report, a subject identification code will be used to link the data to the subject. The code is safeguarded by the principal investigator. During the procedure, all descriptive statistics will be recorded on a paper case-record form. All imaging data for each subject will be stored on a hard drive and locked in room R4.211 of prof. dr. G.M. van Dam, principal investigator. All data will be kept according to institutional guidelines for pathology specimen and study data.

10.2 Amendments

Amendments are changes made to the research after a favorable opinion by the accredited METC has been given. All amendments will be notified to the METC that gave a favorable opinion.

A 'substantial amendment' is defined as an amendment to the terms of the METC application, or to the protocol or any other supporting documentation, that is likely to affect to a significant degree:

1. the safety or physical or mental integrity of the subjects of the trial;
2. the scientific value of the trial;
3. the conduct or management of the trial; or the quality or safety of any intervention used in the trial.

All substantial amendments will be notified to the METC and to the competent authority.

Non-substantial amendments will not be notified to the accredited METC and the competent authority, but will be recorded and filed by the sponsor.

10.3 Annual progress report

The sponsor/investigator will submit a summary of the progress of the trial to the accredited METC once a year. Information will be provided on the date of inclusion of the first subject, numbers of subjects included and numbers of subjects that have completed the trial, serious adverse events/ serious adverse reactions, other problems, and amendments. Additionally, a temporary report will be conducted after the first five patients. This report will be submitted to the METc.

10.4 End of study report

The investigator will notify the accredited METC of the end of the study within a period of 8 weeks. The end of the study is defined as the last participating patient who underwent HIPEC procedure.
In case the study is ended prematurely, the sponsor will notify the accredited METC and the competent authority within 15 days, including the reasons for the premature termination.
Within one year after the end of the study, the investigator/sponsor will submit a final study report with the results of the study, including any publications/abstracts of the study, to the accredited METC.
The sponsor will notify the accredited METC and the competent authority of the end of the study within a period of 90 days. The end of the study is defined as the last patient's surgical procedure.

Within one year after the end of the study, the investigator/sponsor will submit a final study report with the results of the study, including any publications/abstracts of the study, to the accredited METC and the Competent Authority.

10.5 Public disclosure and publication policy

There are no restrictions towards publication; the study will be registered in a public trial registry (www.clinicaltrials.gov).

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