Intraoperative fluorescence imaging in cancer
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INTRAOPERATIVE
TUMOR-SPECIFIC
FLUORESCENCE IMAGING
IN OVARIAN CANCER
BY FOLATE RECEPTOR-
ALPHA TARGETING:
FIRST IN-HUMAN RESULTS
ABSTRACT

BACKGROUND
The prognosis in advanced stage ovarian cancer remains poor. Tumor-specific intraoperative fluorescence imaging may improve staging and debulking efforts in cytoreductive surgery and thereby improve prognosis. The over-expression of folate receptor-alpha (FR-α) in 90-95% of epithelial ovarian cancers prompted the investigation of intraoperative tumor-specific fluorescence imaging in ovarian cancer surgery using an FR-α targeted fluorescent agent.

METHODS
Ten patients with a suspected ovarian malignancy scheduled for an explorative laparotomy were included. Two hours prior to surgery, an intravenous single 10 ml bolus of folate-FITC (0.3 mg/kg) was administered. Multispectral fluorescence imaging was used for the detection of tumor tissue. In vivo images were correlated with histopathological analyses. Ex vivo, the number of tumor spots detected by visual inspection alone was compared to the number detected by fluorescence imaging on still images.

RESULTS
Four patients were diagnosed with a malignant epithelial ovarian tumor and one patient with a borderline tumor. Five patients were diagnosed with a benign ovarian tumor. Fluorescent regions showed excellent correlation with histopathological findings. Ex vivo detection of the number of malignant lesions by fluorescence (median 34, range 8-81) was significantly greater (p<0.001) than by visual observation alone (median 7, range 4–22) in stage III peritoneal carcinomatosis.

CONCLUSIONS
In patients with ovarian cancer, intraoperative tumor-specific fluorescence imaging with a FR-α targeted fluorescent agent showcased the potential applications in patients with ovarian cancer for improved intraoperative staging and more radical cytoreductive surgery.
INTRODUCTION

Of all gynaecologic malignancies, epithelial ovarian cancer (EOC) is the most frequent cause of death, both in the US\(^1\) and in Europe.\(^2\) The relative absence of a clear distinctive clinical presentation in early stages, combined with the lack of a screening tool, often results in disease diagnosis at more advanced stages. The overall 5-year survival rate is 45\%, and only 20-25\% for stage III and IV.\(^4,5\) Currently, cytoreductive surgery, followed by combination chemotherapy is regarded as the most effective treatment. The degree of cytoreduction is one of the few prognostic factors that can be actively influenced by the surgeon, i.e. excision below 1 cm. Improved staging and survival could be achieved by better cytoreduction aided by an intra-operative, tumor-specific detection strategy that assists the surgeon by providing real-time feedback on residual malignant tissue.

Radiologic approaches such as X-ray, CT, MRI or ultrasound have been considered for use in assisting surgical procedures, but these are not tumor-specific and generally not useful for intraoperative applications. In contrast, fluorescence imaging as an optical technique naturally relates to surgical inspection and practice, and it offers superior resolution and sensitivity compared to preoperative radiological imaging and visual inspection and palpation during surgery. The combination of accurate real-time imaging with tumor-specific fluorescent agents can shift the paradigm of surgical inspection by enabling localization of lesions that are difficult or impossible to detect by visual observation or palpation, possibly leading to upstaging of patients due to improved detection of tumor tissue and more radical excision of tumor tissue.

A highly promising target in EOC is the folate receptor-alpha (FR-\(\alpha\)). Recently, several studies on the expression of the FR-\(\alpha\) in ovarian cancer have indicated increased expression in 90-95\% of patients with EOC.\(^6,7\) As such, FR-\(\alpha\) is the ideal target for both therapeutic\(^8-10\) and imaging purposes.\(^11-13\) As a ligand of FR-\(\alpha\), folate has already been conjugated to DTPA for SPECT/CT imaging\(^11\), and to several PET tracers.\(^12\) Moreover, it has been linked to fluorescein for use in imaging metastatic disease in murine tumor models\(^13\), but this was never tested in humans. Using folate conjugated to fluorescein isothiocyanate (folate-FITC), together with a real-time multi-spectral intra-operative fluorescence imaging system\(^14\), we report on the results of first-in-human use of intraoperative tumor-specific fluorescence imaging using a FR-\(\alpha\) targeted fluorescent agent for real-time surgical visualization of tumor tissue in patients undergoing an exploratory laparotomy for suspected ovarian cancer.

METHODS

PATIENTS

The study was approved by the Investigational Review Board (IRB) of the University Medical Center Groningen, the Central Committee on Research involving Human Subjects (CCMO NL26980.042.09; www.ccmo.nl), and registered at the Dutch Trial Reg-
ister (NTR1980; www.trialregister.nl) and the European Clinical Trials Database (EudraCT 2009-010559-29; http://eudract.emea.europa.eu) prior to inclusion. All patients gave their written informed consent and completed the study. Between September 2009 and May 2010, patients scheduled to undergo a staging or debulking laparotomy for suspected ovarian cancer - based on an ovarian mass and/or the presence of ascites as diagnosed by ultrasound or CT scan, and/or elevated CA-125 tumor marker - were consented for the study. Exclusion criteria were: pregnancy, significant renal failure (creatinine > 400 μmol/l), severe cardiac or pulmonary disease (ASA III-IV), a history of iodine allergy or anaphylactic reactions to insect bites or medication and the presence or history of hyperthyroidism. After documented informed consent, the operative procedure was planned. Patients were not limited in their normal behaviour or intake of diet or medication prior to the study. During surgery, patients were staged according to FIGO standard guidelines via a midline laparotomy.

**TUMOR-SPECIFIC FLUORESCENT AGENT**

For targeting of the FR-α in ovarian cancer in patients, the imaging agent was produced according to GMP conditions by Endocyte Inc. (West Lafayette, IN, USA). Folate hapten (vitamin B9) was conjugated with fluorescein isothiocyanate (FITC) yielding folate-FITC (Figure 1A), as described previously by Low et al. Folate-FITC has an excitation wavelength of 495 nm and emits light at 520 nm. The agent specifically targets the FR-α, after which it is internalized into the cytoplasm (Figure 1B).

All vials of folate-FITC were produced according to GMP guidelines and supplied by Endocyte Inc. (folate-FITC produced as EC17) after approval of the IMPD documentation according to FDA/EMEA regulations by the local IRB and Hospital Pharmacy at the University Medical Center Groningen. All imaging agents were stored, recorded and monitored prior to release by the Hospital Pharmacy according to FDA/EMEA and Good Clinical Practice (GCP) guidelines. After intravenous injection of folate-FITC, all patients were monitored during in-hospital admission. Folate-FITC was dissolved in 10 ml sterile normal saline and injected at a dose of 0.3 mg/kg body weight over a period of 10 minutes. Four patients experienced mild discomfort in the upper abdominal region after injection of ~0.1 mg/kg folate-FITC which disappeared within 10-15 minutes, and prompted cessation of the remaining dose according to protocol. All patients completed the entire study. No serious adverse events (SAEs) were reported during or after the study.

**MULTISPECTRAL FLUORESCENT CAMERA SYSTEM**

The camera system was developed by the Technical University Munich / Helmholtz Center Munich (GT, AS, VN). In summary (Figure 2A), the camera system consists of a charge-coupled digital (EM-CCD) camera (Andor Technology, Belfast, UK) for sensitive fluorescence detection, and two separate cameras for detection of intrinsic fluorescence and color (PCO AG, Donaupark, Kelheim, Germany). The system is controlled by a
synchronized multi-CPU computer system (Dell Computer, Round Rock TX, USA) for simultaneous processing of raw data and image registration and rendering. During surgery, the camera was covered with sterile drapes (Carl Zeiss Vision BV, Sliedrecht, the Netherlands, OPMI® Drape REF 306071) (Figure 2B). The system attains a variable field of view (FOV) of 15cm x 15cm to 3cm x 3cm, with a corresponding resolution varying from 150–30 microns. Data are acquired in parallel by all cameras so that color, light attenuation images and fluorescence images are simultaneously collected. Attention has been given to the development of an appropriate multi-spectral strategy that allows color imaging and simultaneous sensitive fluorescence detection in the visible light spectrum, as appropriate for FITC imaging. Besides training of the operating room personnel for covering the camera in sterile draping, no special training is necessary to use the camera intraoperatively.

SURGICAL AND IMAGING PROCEDURE
In all patients a standardized imaging protocol was used prior to, during and after the surgical procedure. On opening the abdominal cavity through a midline incision, the surgeon localized the tumor and inspected and palpated the abdominal and pelvic regions according to FIGO guidelines. At this point, fluorescence video inspection at maximum FOV determined the presence of fluorescence activity, and multi-spectral images were captured (Figure 2C, D+F). Next, the surgeon proceeded with the standard surgical procedure. All tissue suspicious for tumor involvement was imaged both in vivo, and ex vivo immediately after tissue excision, for the presence of fluorescence. Thereafter, the surgical field was inspected with the fluorescence system for remaining fluorescence and additional multi-spectral images were captured. Biopsies were taken of residual fluorescent tissue, being part of the standard operative procedure. For ethical reasons, it was decided to remove fluorescent tissue if it did not impose an additive negative effect on morbidity as judged by the operating surgeon.

DETECTION OF TUMOR DEPOSITS BY FLUORESCENCE IMAGING
Still images (color and fluorescence) of three abdominal regions were copied from the collected video data on the patient with extensive, FR-α positive, peritoneal carcinomatosis (stage III). Five surgeons, blinded for the surgical procedure and the final histopathological status of the removed tissue, were asked to identify tumor deposits based on the color images and their corresponding fluorescence images. They independently received a sealed envelope with three plasticized full-color images (Figure 3A). Next, they identified suspicious tumor deposits using a permanent marker. After collecting the first set of envelopes, the same surgeons independently received a second sealed envelope with three plasticized corresponding fluorescence images with exactly the same anatomical location (Figure 3B). Again, they identified tumor deposits, now guided by fluorescence; i.e white spots against a black background, by using a permanent marker.
HISTOPATHOLOGICAL ANALYSES
All specimens were examined by one pathologist (JB) using standard hematoxylin/eosin (H/E) staining. Additionally, immunohistochemical (IHC) staining for FR-α was performed by a standard protocol as described by Low et al. using monoclonal antibody mAb343 (kindly provided by Dr. Wilbur Franklin, University of Colorado Health Science Center, Denver, CO, USA). Kidney samples and placental tissue served as positive controls. All samples were semi-quantitatively scored according to previously reported criteria. All slices were examined with a light microscope (Leica DM4000B, Leica Microsystems BV, Rijswijk NL). Microphotographs were taken with a Leica microscope camera (Leica DFC320, Leica Microsystems BV, Rijswijk NL). Additionally, slices of all excised tissue were examined by fluorescence microscopy (Zeiss Axioplan 2 with Axiocam camera system and Axiovision software, version 4.6.3.0, Carl Zeiss BV, Sliedrecht NL) in the FITC channel (495/520 nm).

STATISTICAL ANALYSES
The number of tumor deposits on the color image was compared pair wise to the number identified by intraoperative tumor-specific fluorescence imaging (non-parametric Wilcoxon signed rank test). A p-value of <0.05 was considered statistically significant.

RESULTS

PATIENT CHARACTERISTICS
The mean age of all patients was 61.2 ± 11.4 (SD). Four patients were diagnosed with a malignant epithelial ovarian tumor (two serous carcinomas, one undifferentiated carcinoma and one mucinous carcinoma) and one patient with a serous borderline tumor. Five patients were diagnosed with a benign ovarian tumor, as confirmed by histopathology: two fibrothecomas, one cellular fibroma, one cystic teratoma and one benign multicystic ischemic ovary (Table 1).

INTRAOPERATIVE TUMOR-SPECIFIC FLUORESCENCE IMAGING
Use of the intraoperative imaging system did not interfere with the standard surgical procedure (Supplemental Video). The mean duration for capturing in vivo intraoperative fluorescence images and video was 10 minutes (range: 4-36 minutes). Fluorescence was detectable intraoperatively in all patients with a malignant tumor and FR-α expression, whereas in the patient with a malignant tumor and those with a benign tumor and no FR-α expression, fluorescence was absent (Table 1). Healthy tissue did not show any fluorescence signal in vivo, nor ex vivo or on histopathological validation. In two separate still images of patients with ovarian cancer, the mean tumor-to-background ratio (TBR) for 10 demarcated fluorescent tumor deposits in each still image was 3.1 (± 0.8 SD), compared to healthy peritoneal surface (data not shown). In the patient with a
high-grade serous carcinoma and extensive peritoneal disseminated disease (stage III, FR-α positive), widespread tumor-specific fluorescence (white spots) was present throughout the abdominal cavity (Figure 2E+G), as confirmed by ex vivo histopathology. Real-time image-guided excision of fluorescent tumor deposits with a size smaller than 1 mm was feasible (Figure 3) and all fluorescent tissue was confirmed to be malignant by histopathology. In the same patient, the tumor-specific fluorescent signal originating from disseminated tumor deposits could be detected up to 8 hours post-injection during a prolonged procedure.

DETECTION OF TUMOR DEPOSITS BY FLUORESCENCE EX VIVO
Five surgeons independently identified tumor deposits on three separate color images (shown on a representative image in Figure 4A) and on their corresponding fluorescence image of precisely the same area (Figure 4B). Detection of the number of tumor deposits guided by tumor-specific fluorescence (median 34, range 8-81) was significantly higher compared to visual observation alone (median 7, range 4-22, p<0.001) (Figure 4C).

POST-OPERATIVE HISTOPATHOLOGICAL ANALYSES
All excised fluorescent tissue was again analyzed for tumor-specific fluorescence ex vivo, in which tumor deposits could be visualized with a resolution of approximately ≤ 1.0 mm (Figure 3).
Representative examples of postoperative histopathological analyses are depicted in Figure 5 for three different ovarian tumors (fibrothecoma (A), serous borderline tumor (B), and high grade serous carcinoma (C)). Routine histopathological examination using hematoxylin/eosin (H/E) staining was performed to determine the nature of the excised tissue (Figure 5, top row). All fluorescent tissue samples were confirmed to contain tumor, whereas non-fluorescent tissue was free of tumor. Additionally, immunohistochemical (IHC) staining for FR-α revealed strong expression in the malignant tumors, moderate expression in the borderline tumor and no expression in the benign lesions (Figure 5, middle row). On one of the benign lesions there was considerable inflammation with increased number of macrophages. This patient showed no fluorescence activity in vivo, nor ex vivo by fluorescence microscopy (data not shown). Finally, fluorescence microscopy for folate-FITC showed a strong signal in all malignant tumors with FR-α expression and no signal in FR-α negative malignant or benign lesions (Figure 5, bottom row). These results correlate excellent with the presence and intensity of the intraoperative fluorescence signal (Table 1).
DISCUSSION
Ovarian cancer, known as the ‘silent lady killer’, is the leading cause of death among gynecologic malignancies in the western world. Because of late onset of symptoms, 75% of patients are diagnosed with advanced disease (stage III and IV), with a 5-year survival rate of 20%. The current treatment of choice is optimal cytoreductive surgery, followed by chemotherapy in stages II and higher. In this proof-of-concept study, we investigated the potential value of intraoperative tumor-specific fluorescence imaging in the detection of tumor tissue in patients with ovarian cancer.

In this limited series, we showed that the use of intraoperative tumor-specific fluorescence imaging of the systemically administered FR-α targeted agent folate-FITC offers specific and sensitive real-time identification of tumor tissue during surgery in patients with ovarian cancer and the presence of FR-α expression. A significantly higher number of fluorescent tumor deposits was detected compared to conventional visual inspection in a patient with extensive peritoneal carcinomatosis. Although the scoring was not accompanied by tactile information, we feel confident to compare the two imaging methods (i.e. visual observation alone vs. fluorescence imaging) alone, instead of adding tactile information, of which a gold standard is lacking. Although tactile information is considered by many surgeons as an important feature in staging, clear data is lacking in literature on the exact sensitivity, specificity and diagnostic accuracy of palpation in cancer surgery. Our finding makes it potentially even more useful in laparoscopic staging of ovarian cancer, which lacks by definition the surgeons direct manual tactile information. Currently, we are in the process of also developing laparoscopic fluorescence camera systems for this particular purpose. So far, this technique did not demonstrate unwanted interference with standard surgical procedures.

In this pilot study, folate-FITC in an intravenously injected formulation appears to be safe. Furthermore, it has a pharmacodynamic profile that facilitates fluorescence imaging over the course of two hours up to eight hours upon injection. When injected two hours prior to surgery, folate-FITC yields a clearly discernible signal in FR-α positive tumor tissue. This systemic administration of a targeted fluorescent agent in humans indicates a versatile platform for intraoperative tumor-specific fluorescence imaging.

The use of targeted fluorescent agents can offer a paradigm shift in surgical imaging as it allows an engineered approach to improve staging and the technique of cytoreductive surgery and thereby improving the outcome in ovarian cancer. A major advantage over current imaging modalities is that an intraoperative fluorescence imaging system offers a large field of view for inspection and staging. Additionally, high resolution images are acquired through optical zoom and magnification for highly accurate identification of local tumor deposits. This in turn can enable future patient-tailored surgical interventions and may decrease the number of needless extensive surgical procedures, and in turn, decrease associated morbidity. Besides improved staging, primarily anticipated in stage I and II, the second major advantage of intraoperative imaging above current standards is that it may guide the surgeon in debulking efforts, thus contributing to more efficient...
cytoreduction and ultimately improving the effect of adjuvant chemotherapy in patients with reduced tumor load. Since cytoreduction is a key aspect in the prognosis of many solid tumors with a peritoneal dissemination pattern, including ovarian cancer\textsuperscript{20,21} and colorectal cancer\textsuperscript{22}, a more complete debulking procedure may have a beneficial effect on outcome in different types of cancer. Improving the detection of cancer deposits to submillimeter size might ultimately improve survival rates, but needs to be established by additional clinical studies.

A crucial aspect in intraoperative tumor-specific fluorescence imaging is the identification and validation of a tumor-specific target. In ovarian cancer, the FR-\(\alpha\) appears to constitute an ideal target, since it is over-expressed in 90-95\% of malignant tumors, especially serous carcinomas. Furthermore, the targeting ligand, folate, is attractive as it is non-toxic, inexpensive and relatively easily conjugated to a fluorescent dye to create a tumor-specific fluorescent contrast agent.\textsuperscript{23} However, over-expression of FR-\(\alpha\) varies strongly between different solid tumors originating from different organs\textsuperscript{24}, thus reducing the general applicability of folate-FITC in cancer. Future developments in identification of tumor-specific targets will undoubtedly bridge this gap and open new revolutionary possibilities for intraoperative tumor-specific fluorescence imaging in cancer surgery for other solid tumors. Subsequently, development of new fluorescent agents in the near-infrared spectrum will allow for identification of deeper seated tumors, based on the stronger penetration properties of near-infrared dyes with an excitation wavelength >700 nm compared to FITC.\textsuperscript{25} As an add-on, targeted imaging will not only provide a diagnostic and image-guidance tool to the surgeon, but also provide information of the target for (neo-)adjuvant targeted therapeutics.

In summary, our data outline the first in-human proof-of-principle and the potential benefit of intraoperative tumor-specific fluorescence imaging in staging and debulking surgery for ovarian cancer using the systemically administered targeted fluorescent agent, folate-FITC. The combination of state-of-the-art optical imaging technologies with sophisticated targeting strategies can shift the paradigm of surgical oncologic imaging, offering the unique opportunity to intraoperatively detect and quantify tumor growth and intra-abdominal spread. We are currently planning a larger international multicenter study using standardized, uniformly calibrated multispectral fluorescence camera systems as described in the paper combined with folate-FITC to confirm our data and further elucidate the diagnostic (accuracy, sensitivity and specificity) and therapeutic value of the reported approach in larger series of ovarian cancer patients.

**ACKNOWLEDGEMENTS**

The authors wish to thank dr. Wim Sluiter for help with statistical analyses, Mrs Ina Wesselman for training the operating room personnel in using the camera system and dr. Barbara Molmans (Hospital Pharmacy) for pharmaceutical expertise and support.
### Table 1 – Demographics and individual data of patients

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+++ = strong; + = moderate; 0 = weak; - = absent
FIGO = International Federation of Gynecology and Obstetrics
IHC FR-α = immunohistochemistry folate-receptor alpha
FM FITC = fluorescence microscopy for folate-FITC, n.a. = not applicable
REFERENCE LIST


