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FLUORESCENCE-GUIDED ENDOSCOPY AND SURGERY

Clinical applications for optical imaging agents

Steven Jakob de Jongh

S.J. de Jongh
FLUORESCENCE-GUIDED ENDOSCOPY AND SURGERY
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FLUORESCENCE-GUIDED ENDOSCOPY AND SURGERY

Clinical applications for optical imaging agents

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ter verkrijging van de graad van doctor aan de
Rijksuniversiteit Groningen
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General introduction and outline of the thesis

GENERAL INTRODUCTION

Cancer is one of the leading causes of death worldwide and with approximately 18.1 million new cases in 2018, the incidence is still rapidly increasing.¹ Cancer of the digestive system is the most common form of cancer worldwide.¹ This thesis focusses on the (early) detection and treatment of three types of solid cancers of the digestive system and their precursor lesions: oral cavity cancer, esophageal cancer and colorectal cancer.

Incidence and treatment

Oral cavity cancer

Cancer of the oral cavity accounts for approximately 2% of all cancer-related deaths worldwide. With primary etiological factors being tobacco and alcohol use, the geographical incidence tends to cluster in Southern Asia, although oral cavity cancers are quite common in more developed countries as well.^{1,2} The main histological subtype is squamous cell carcinoma, which accounts for almost 90% of oral cavity cancers.³ Primary surgical resection is one of the main treatments for head and neck squamous cell carcinomas (HNSCC), which may be combined with radiation therapy, chemotherapy or targeted therapy depending on the anatomical location and stage of the disease.³

Esophageal cancer

Esophageal cancer is the seventh most common cancer type worldwide and causes 5.3% of cancer-related deaths.¹ There are two main histological subtypes with a different etiology and geographical incidence: esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma. EAC has the highest incidence in the Western world and is predominantly caused by obesity and gastro-esophageal reflux disease. In contrast, esophageal squamous cell carcinomas particularly appear in Asian countries and although etiological factors are not yet fully understood, smoking and drinking seem to play a major role.¹

Malignant degeneration to EAC is a multi-step process, which commonly starts with a metaplastic change of the esophageal mucosal cell lining to intestinal metaplasia, i.e. Barrett's esophagus (BE).^{4,5} BE patients undergo surveillance high-definition white-light endoscopies (HD-WLE) with random four-quadrant biopsies every 1-2 centimeters of the BE-segment. However, small and flat lesions with subtle morphological characteristics can easily be missed and random biopsies pose a high change of sampling error, resulting in a detection miss-rate of up to 57%.^{6,7} Neoplastic lesions in BE-patients can be removed relatively safely using an endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD), with promising treatment outcomes that improve survival.^{8,9} When endoscopic treatment is not feasible due to advanced tumor growth into the submucosa, an esophagectomy combined with neoadjuvant chemoradiotherapy is performed, with a significant 33.1% morbidity and a perioperative mortality rate of 3.1%. Early detection and removal of neoplastic BE-lesions therefore is essential to prevent such extensive surgical procedures, which are associated with a decreased quality of life.¹⁰

Colorectal cancer

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths worldwide, accounting for nearly 1 in 10 cancer deaths each year.¹ Similar to EACs, the majority of CRCs are thought to develop through the adenoma-to-carcinoma sequence, emphasizing the need for early detection of colorectal polyps that bear a malignant potential, such as adenomas and sessile serrated polyps.¹¹ HD-WLE is the gold standard for detection and removal of colorectal polyps and has significantly improved the colorectal detection rate. However, a detection miss-rate of up to 22% remains, particularly for right-sided and smaller polyps, due to factors such as inadequate bowel preparation and the skill of the endoscopist.^{12–14}

The main treatment pillar for CRC treatment is surgical resection, as around 90% of colon cancers and 84% of rectal cancers are treated surgically.¹⁵ Colon cancers are situated in the large intestine up to 12 cm from the anal verge, from which they are defined as rectal cancers. Depending on preoperative tumor-node-metastases (TNM) staging, (neoadjuvant) chemoradiotherapy also has a role in CRC treatment.^{15,16}

Approximately 3–21% of CRC patients develop metastases limited to the peritoneum, i.e. peritoneal carcinomatosis (PC).¹⁷ Patients with PC of colorectal origin can be treated with cytoreductive surgery to remove all macroscopic tumor deposits, combined with hyperthermic intraperitoneal chemotherapy to eradicate the remaining microscopic disease.¹⁸

Clinical challenges in gastroenterology

In gastroenterology, early detection, characterization and removal of (pre)malignant lesions is crucial. However, a considerable number of lesions is still being missed in daily clinical practice^{6,7,12,13}, while the therapeutic capabilities of the endoscopist are only expanding due to technological improvements in advanced endoscopic therapies such as EMR, ESD or endoscopic full-thickness resection. This has created a shift in the treatment of gastrointestinal cancers that are limited to the submucosa, from a mainly surgical to a more minimal invasive, interventional gastroenterology approach. Overall, this emphasizes the need for early detection of (pre)malignant lesions in the gastrointestinal tract even more, in order to improve outcome and prognosis.¹⁹

As a result, several advanced endoscopic imaging techniques have been developed and translated into the clinic, such as narrow-band imaging, optical chromoendoscopy and autofluorescence imaging.²⁰ The majority of these techniques are applied complementary to HD-WLE, using an integrated or fiber-based approach. In general, contrast is provided by enhancing morphological characteristics of aberrant mucosal lesions during endoscopy, for example through application of non-specific contrast agents or technological features that are based on the use of specific wavelengths.²⁰ However, for CRC and EAC screening purposes, none of these techniques have significantly contributed to improved lesion detection rates. Therefore, there is an unmet need for a novel endoscopic imaging technique that has the potential to improve endoscopic lesion detection rates.

Clinical challenges in surgical oncology

In surgical oncology, complete removal of all tumor tissue, i.e. a tumor-negative resection margin, is of paramount importance for treatment outcome and survival. Currently, the gold standard for discriminating between tumor and benign tissue is visual inspection and palpation, which is highly dependent on the tissue characteristics and the experience of a surgeon. Moreover, this becomes increasingly difficult when the tissue architecture has changed due to neoadjuvant chemoradiotherapy or scarring from previous surgeries.²¹

Consequently, tumor-positive resection margins are far too common, as for example 21-40% of head and neck cancer surgeries^{22,23} and approximately 18.6% of locally advanced rectal cancer surgeries^{24,25} prove to have a tumor-positive resection margin after histopathological examination. This is highly relevant, as tumor-negative resection margins reduce the need for adjuvant treatment, decrease the risk of local recurrence and consequently improve survival.^{26,27} Also for PC, where the extent of the disease and the completeness of cytoreductive surgery are the most important prognostic factors, complete tumor removal is crucial for patient prognosis.²⁸

When in doubt, intraoperative frozen section analysis can be performed to evaluate the resection margin status, despite the fact this is time-consuming and poses a high risk of sampling error.^{29,30} Currently, there are no other clinically approved techniques available that can assist in intraoperative cancer detection and resection margin evaluation. Therefore, there is an unmet need for a technique that can improve *real-time* intraoperative cancer detection, in order to remove all tumor tissue and improve tumor-negative resection margin rates.

The principles of optical molecular imaging using fluorescence

During the course of a disease, numerous biomarkers become overexpressed or upregulated, such as receptors or proteins. Optical molecular imaging is an emerging technique that generates contrast by specifically visualizing these biomarker expression levels in *real-time*, using targeted (near-)infrared fluorescent imaging agents. Consequently, this technique has the potential to serve as a 'red-flag' endoscopic or intraoperative imaging modality that may enable the differentiation between (pre)malignant lesions and normal tissue.^{31,32}

The principle of fluorescence imaging is based on excitation of a fluorophore that subsequently emits light of a longer wavelength, which can be detected using a fluorescence camera. Fluorescence imaging is influenced by several factors, among which the tissue optical properties (i.e. scattering and absorption). The near-infrared (NIR) light spectrum (650-900 nm) provides favorable properties for fluorescence imaging, as it has a relatively low tissue absorption and therefore a high penetration depth of up to ± 1 cm, and relatively low autofluorescence.³³ Sensitive fluorescence camera systems are required to visualize NIR fluorescence during endoscopy or surgery. The University Medical Center Groningen has collaborated with the Technical University of Munich to develop a fiber-based endoscopic fluorescence camera system and a wide-field fluorescence camera for intraoperative use, which have been further developed commercially.^{34,35}

AIMS AND OUTLINE OF THE THESIS

The main aim of this thesis is to improve (pre)malignant lesion detection during endoscopy and to improve intraoperative tumor detection during surgery, through clinical application of targeted NIR fluorescent imaging agents. This thesis describes the clinical application of three targeted fluorescent imaging agents for different indications: EMI-137³⁶ targeting c-Met, bevacizumab-800CW³⁷ targeting the vascular endothelial growth factor A (VEGFA) and cetuximab-800CW³⁸ targeting the epidermal growth factor epidermal growth factor receptor (EGFR).

Part I of this thesis describes the clinical application of targeted fluorescence imaging in endoscopy, i.e. fluorescence molecular endoscopy (FME). In **Chapter 2**, we review the mechanism of action and clinical status of currently available endoscopic imaging enhancement techniques that aim to improve the diagnostic and minimal invasive therapeutic options for a gastroenterologist. Endoscopic imaging enhancement techniques can roughly be classified into two categories: wide-field imaging techniques and cross-sectional imaging techniques. Wide-field imaging techniques enable the macroscopic evaluation of a relatively large mucosal surface, while cross-sectional imaging techniques typically provide more microscopic details by facilitating virtual histology.

One of these wide-field imaging techniques is FME. FME was previously shown to potentially improve the colorectal polyp detection rate using the fluorescent peptide EMI-137, targeting the c-Met membrane receptor.³⁶ However, the investigated dose-to-imaging interval of three hours might hamper further clinical application in a screening population. In **Chapter 3**, we describe a clinical study that investigates the optimal dose-to-imaging interval for FME using EMI-137 for colorectal polyp detection. Fluorescence was visualized using FME after a one-, two- and three-hour dose-to-imaging interval. However, fluorescence intensities are influenced by several factors, such as the imaging distance, illumination homogeneity and tissue optical properties like scattering and absorption. To correct for these factors and confirm *in vivo* FME results, we subsequently quantified intrinsic fluorescence values using direct contact multi-diameter single-fiber reflectance, single-fiber fluorescence (MDSFR/SFF) spectroscopy measurements.

Several clinical FME safety and feasibility studies have been performed over the past decade, investigating different optical imaging agents.^{36,40–44} However, as FME is an emerging technique, there are no standards or guidelines that define FME study objectives, outcome parameters or imaging specifications. **Chapter 4** describes a clinical study investigating the safety, feasibility and optimal method of administration of EMI-137 during FME in patients with Barrett's neoplasia, using *in vivo* FME, *in vivo* quantification of fluorescence by MDSFR/SFF spectroscopy and an extensive *ex vivo* validation. In addition, **Chapter 4** also describes a structured methodology and six important outcome parameters, in order to guide the validation of a fluorescent tracer in future early phase clinical FME studies.

Part II of this thesis describes the clinical application of targeted fluorescence imaging during surgery, i.e. fluorescence-guided surgery (FGS), and pathology. In **Chapter 5**, we explain the principles of intraoperative optical molecular imaging and provide a chronological overview of the development of FGS in patients with PC of CRC. In addition, we review biomarker selection criteria, describe potential targets for CRC imaging and the optical imaging agents that can potentially benefit CRC and colorectal PC detection.

Additionally, **Chapter 6** describes the first in-human phase-I clinical feasibility study that evaluated the NIR fluorescent tracer bevacizumab-800CW, targeting the vascular endothelial growth factor-A (VEGFA), for the detection of colorectal PC. VEGFA is an angiogenic growth factor that is known to be overexpressed in approximately 93% of colorectal peritoneal metastases.⁴⁵ We evaluated the safety and potential clinical added value of FGS for improved *real-time* tumor detection, which was validated by extensive *ex vivo* correlation between fluorescence intensities, histology and VEGFA expression levels, from a macroscopic to microscopic level.

In **Chapter 7**, we describe a quantitative dose-escalation study investigating another NIR fluorescent tracer, cetuximab-800CW, for improved resection margin evaluation in patients with HNSCC. Cetuximab targets the cell-membrane receptor epidermal growth factor receptor (EGFR), which is overexpressed in around 90% of HNSCCs.⁴⁶ In this safety and dose-escalation study, we evaluate three different single flat-doses of cetuximab-800CW and two doses of cetuximab-800CW preceded by an unlabeled pre-dose of cetuximab. Moreover, we demonstrate that quantification of intrinsic fluorescence values using MDSFR/SFF spectroscopy contributes to determining the optimal tracer dose.

In **Chapter 8**, we describe a retrospective proof-of-concept study, investigating the feasibility of back-table fluorescence-guided imaging (FGI) for evaluation of the resection margin status of locally advanced rectal cancer (LARC) patients at the operating theatre, using bevacizumab-800CW. Currently, the gold standard for evaluating the resection margins status after surgery is histopathological examination, which can take up to seven days. We analyzed back-table FGI data of fresh surgical specimens of LARC patients that were enrolled in a clinical trial evaluating VEGFA-targeted FME.⁴⁴ We validated the fluorescence imaging results by extensive *ex vivo* analyses. In addition, we provide a standardized methodology for data collection and analysis for future studies, based on our experience from this study, **Chapter 4** and a recently reported analytical framework.⁴⁷

Finally, **Chapter 9** summarizes and discusses the findings of this thesis and the future perspectives for FME and FGS.

REFERENCES

- 1 Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *Ca Cancer J Clin* 2018; 68: 394–424.
- 2 Gandini S, Botteri E, Iodice S, et al. Tobacco smoking and cancer: A meta-analysis. *Int J Cancer* 2008; 122: 155–64.
- 3 Montero PH, Patel SG. Cancer of the Oral Cavity. *Surg Oncol Clin N Am* 2015; 24: 491–508.
- 4 Burke ZD, Tosh D. Barrett's metaplasia as a paradigm for understanding the development of cancer. *Curr Opin Genet Dev* 2012; 22: 494–9.
- 5 Morales CP, Souza RF, Spechler SJ. Hallmarks of cancer progression in Barrett's oesophagus. *Lancet* 2002; 360: 1587–9.
- 6 Vieth M, Ell C, Gossner L, May A, Stolte M. Histological Analysis of Endoscopic Resection Specimens From 326 Patients with Barrett's Esophagus and Early Neoplasia. *Endoscopy* 2004; 36: 776–81.
- 7 Shaheen NJ, Falk GW, Iyer PG, Gerson LB. ACG Clinical Guideline: Diagnosis and Management of Barrett's Esophagus. *Am J Gastroenterology* 2016; 111: 30.
- 8 Tomizawa Y, Iyer PG, Song LMWK, Buttar NS, Lutzke LS, Wang KK. Safety of Endoscopic Mucosal Resection for Barrett's Esophagus. *Am J Gastroenterology* 2013; 108: 1440.
- 9 Haidry RJ, Dunn JM, Butt MA, et al. Radiofrequency Ablation and Endoscopic Mucosal Resection for Dysplastic Barrett's Esophagus and Early Esophageal Adenocarcinoma: Outcomes of the UK National Halo RFA Registry. *Gastroenterology* 2013; 145: 87–95.
- 10 Raymond DP, Seder CW, Wright CD, et al. Predictors of Major Morbidity or Mortality After Resection for Esophageal Cancer: A Society of Thoracic Surgeons General Thoracic Surgery Database Risk Adjustment Model. *Ann Thorac Surg* 2016; 102: 207–14.
- 11 Leslie A, Carey FA, Pratt NR, Steele RJC. The colorectal adenoma–carcinoma sequence. *Brit J Surg* 2002; 89: 845–60.
- 12 Atkin W, Wooldrage K, Parkin DM, et al. Long term effects of once-only flexible sigmoidoscopy screening after 17 years of follow-up: the UK Flexible Sigmoidoscopy Screening randomised controlled trial. *Lancet* 2017; 389: 1299–311.
- 13 Rijn JC van, Reitsma JB, Stoker J, Bossuyt PM, Deventer SJ van, Dekker E. Polyp Miss Rate Determined by Tandem Colonoscopy: A Systematic Review. *Am J Gastroenterology* 2006; 101: 343–50.
- 14 Bressler B, Paszat LF, Vinden C, Li C, He J, Rabeneck L. Colonoscopic miss rates for right-sided colon cancer: A population-based analysis. *Gastroenterology* 2004; 127: 452–6.
- 15 Nelson H, Petrelli N, Carlin A, et al. Guidelines 2000 for Colon and Rectal Cancer Surgery. *Jnci J National Cancer Inst* 2001; 93: 583–96.
- 16 Klaver CEL, Wisselink DD, Punt CJA, et al. Adjuvant hyperthermic intraperitoneal chemotherapy in patients with locally advanced colon cancer (COLOPEC): a multicentre, open-label, randomised trial. *Lancet Gastroenterol Hepatol* 2019; 4: 761–770.
- 17 Koppe MJ, Boerman OC, Oyen WJG, Bleichrodt RP. Peritoneal Carcinomatosis of Colorectal Origin. *Ann Surg* 2006; 243: 212–22.
- 18 Verwaal VJ, Bruin S, Boot H, Slooten G van, Tinteren H van. 8-Year Follow-up of Randomized Trial: Cytoreduction and Hyperthermic Intraperitoneal Chemotherapy Versus Systemic Chemotherapy in Patients with Peritoneal Carcinomatosis of Colorectal Cancer. *Ann Surg Oncol* 2008; 15: 2426–32.
- 19 Kiesslich R, Goetz M, Hoffman A, Galle PR. New imaging techniques and opportunities in endoscopy. *Nat Rev Gastroenterol* 2011; 8: 547.
- 20 East JE, Vleugels JL, Roelandt P, et al. Advanced endoscopic imaging: European Society of Gastrointestinal Endoscopy (ESGE) Technology Review. *Endoscopy* 2016; 48: 1029–45.
- 21 Tanaka S, Martling A, Lindholm J, Holm T, Palmer G. Remaining cancer cells within the fibrosis after neo-adjuvant treatment for locally advanced rectal cancer. *European J Surg Oncol Ejs* 2015; 41: 1204–9.
- 22 Woolgar JA, Triantafyllou A. A histopathological appraisal of surgical margins in oral and oropharyngeal cancer resection specimens. *Oral Oncol* 2005; 41: 1034–43.
- 23 Smits RWH, Koljenović S, Hardillo JA, et al. Resection margins in oral cancer surgery: Room for improvement. *Head Neck* 2016; 38: E2197–203.

- 24 Simillis C, Baird DLH, Kontovounisios C, et al. A Systematic Review to Assess Resection Margin Status After Abdominoperineal Excision and Pelvic Exenteration for Rectal Cancer. *Ann Surg* 2017; 265: 291–9.
- 25 MERCURY study group. Diagnostic accuracy of preoperative magnetic resonance imaging in predicting curative resection of rectal cancer: prospective observational study. *Bmj* 2006; 333:779.
- 26 Sutton DN, Brown JS, Rogers SN, Vaughan ED, Woolgar JA. The prognostic implications of the surgical margin in oral squamous cell carcinoma. *Int J Oral Max Surg* 2003; 32: 30–4.
- 27 Nagtegaal ID, Quirke P. What Is the Role for the Circumferential Margin in the Modern Treatment of Rectal Cancer? *J Clin Oncol* 2008; 26: 303–12.
- 28 Sugarbaker PH, Ryan DP . Cytorreductive surgery plus hyperthermic perioperative chemotherapy to treat peritoneal metastases from colorectal cancer: standard of care or an experimental approach? *Lancet Oncol* 2012; 13: e362–9.
- 29 DiNardo LJ, Lin J, Karageorge LS, Powers CN. Accuracy, Utility, and Cost of Frozen Section Margins in Head and Neck Cancer Surgery. *Laryngoscope* 2000; 110: 1773–6.
- 30 Khoury W, Abboud W, HersHKovitz D, Duek SD. Frozen section examination may facilitate reconstructive surgery for mid and low rectal cancer. *J Surg Oncol* 2014; 110: 997–1001.
- 31 Sommen F van der, Curvers WL, Nagengast WB. Novel developments in endoscopic mucosal imaging. *Gastroenterology* 2018; 154: 1876–86.
- 32 Boer E de, Harlaar NJ, Taruttis A, et al. Optical innovations in surgery. *Brit J Surg* 2015; 102: e56–72.
- 33 Weissleder R, Ntziachristos V. Shedding light onto live molecular targets. *Nat Med* 2003; 9: 123–8.
- 34 Themelis G, Yoo JS, Soh K-S, Schulz R, Ntziachristos V . Real-time intraoperative fluorescence imaging system using light-absorption correction. *J Biomed Opt* 2009; 14: 064012-064012–9.
- 35 Garcia-Allende PB, Glatz J, Koch M, et al. Towards clinically translatable NIR fluorescence molecular guidance for colonoscopy. *Biomed Opt Express* 2013; 5: 78–92.
- 36 Burggraaf J, Kamerling IMC, Gordon PB, et al. Detection of colorectal polyps in humans using an intravenously administered fluorescent peptide targeted against c-Met. *Nat Med* 2015; 21: 955–61.
- 37 Weele EJ ter, Scheltinga AGTT van, Linssen MD, et al. Development, preclinical safety, formulation, and stability of clinical grade bevacizumab- 800CW, a new near infrared fluorescent imaging agent for first in human use. *Eur J Pharm Biopharm* 2016; 104: 226–34.
- 38 Linssen MD, Weele EJ ter, Allersma DP, et al. Roadmap for the Development and Clinical Translation of Optical Tracers Cetuximab-800CW and Trastuzumab-800CW. *J Nucl Med* 2019; 60: 418–23.
- 39 Bird-Lieberman EL, Neves AA, Lao-Sirieix P, et al. Molecular imaging using fluorescent lectins permits rapid endoscopic identification of dysplasia in Barrett's esophagus. *Nat Med* 2012; 18: 315.
- 40 Nagengast WB, Hartmans E, Garcia-Allende PB, et al. Near-infrared fluorescence molecular endoscopy detects dysplastic oesophageal lesions using topical and systemic tracer of vascular endothelial growth factor A. *Gut* 2019; 68: 7.
- 41 Hartmans E, Tjalma JJJ, Linssen MD, et al. Potential Red-Flag Identification of Colorectal Adenomas with Wide-Field Fluorescence Molecular Endoscopy. *Theranostics* 2018; 8: 1458–67.
- 42 Joshi BP, Dai Z, Gao Z, et al. Detection of Sessile Serrated Adenomas in the Proximal Colon Using Wide-Field Fluorescence Endoscopy. *Gastroenterology* 2017; 152: 1002–13.
- 43 Atreya R, Neumann H, Neufert C, et al. In vivo imaging using fluorescent antibodies to tumor necrosis factor predicts therapeutic response in Crohn's disease. *Nat Med* 2014; 20: nm.3462.
- 44 Tjalma JJ, Koller M, Linssen MD, et al. Quantitative fluorescence endoscopy: an innovative endoscopy approach to evaluate neoadjuvant treatment response in locally advanced rectal cancer. *Gut* 2019; : 1-5 Epub ahead of print.
- 45 Cuba EMV de, Hingh IHJT de, Sluiter NR, et al. Angiogenesis-Related Markers and Prognosis After Cytorreductive Surgery and Hyperthermic Intraperitoneal Chemotherapy for Metastatic Colorectal Cancer. *Ann Surg Oncol* 2016; 23: 1601–8.
- 46 Grandis JR, Tweardy DJ. Elevated levels of transforming growth factor alpha and epidermal growth factor receptor messenger RNA are early

markers of carcinogenesis in head and neck cancer. *Cancer Res* 1993; 53: 3579–84.

- 47 Koller M, Qiu S-Q, Linssen MD, et al. Implementation and benchmarking of a novel analytical framework to clinically evaluate tumor-specific fluorescent tracers. *Nat Commun* 2018; 9: 3739.

PART I

FLUORESCENCE-GUIDED ENDOSCOPY

2

Endoscopic imaging enhancement techniques

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ABSTRACT

Recent advances in the field of endoscopy have led to the development of several novel imaging techniques that aim to improve and expand the diagnostic and therapeutic capabilities of an endoscopist. Roughly, endoscopic imaging enhancement techniques can be divided into two categories: wide-field imaging techniques and cross-sectional imaging techniques. This review aims to provide an overview of the mechanism of action and clinical status of different endoscopic imaging enhancement techniques that may be used to overcome current clinical challenges in mucosal lesion detection.

INTRODUCTION

In current clinical practice, high definition white-light endoscopy (HD-WLE) is the standard of care for diagnosis and treatment monitoring of many mucosal diseases in gastroenterology. For instance, it is regarded as the gold standard for the detection of colorectal polyps during screening programs and for the detection of esophageal neoplasia during surveillance endoscopies in patients with Barrett's esophagus (BE).

Although HD-WLE has contributed to an increased detection rate of (pre)malignant mucosal lesions, gastrointestinal cancer still remains the second leading cause of cancer-related deaths worldwide (Siegel et al., 2017). Partly this is caused by missing lesions during surveillance endoscopies. For instance, $\pm 10\%$ – 25% of all colorectal adenomas remain undetected during standard HD-colonoscopy. In the majority of cases this concerns sessile, flat or right-sided polyps (Heresbach et al., 2008; Kaminski et al., 2010; van Rijn et al., 2006). Also, in patients with BE, dysplasia and early adenocarcinoma can easily be missed during endoscopic examination, as subtle mucosal changes are difficult to detect (Sharma et al., 2013).

To a certain extent, miss-rates can be explained by factors such as poor bowel preparation and (the lack of) experience of an endoscopist. However, also technical factors such as limited contrast and resolution may play a role in missing small or flat lesions (Barclay et al., 2006). As earlier detection of (pre)malignant lesions may improve survival for upper and lower gastrointestinal malignancies by facilitating (earlier) endoscopic treatment, there is a need for improved endoscopic imaging techniques that expands the diagnostic and therapeutic capabilities of the endoscopist. Recent technological improvements in optics, filters, and processors alongside with higher resolution and advanced software have already led to the development of several techniques that may improve the detection of early neoplastic lesions. These endoscopic imaging enhancement techniques can roughly be divided into two categories: wide-field imaging techniques and cross-sectional imaging techniques.

Wide-field imaging techniques such as optical chromoendoscopy (CE), narrow band imaging (NBI) or auto-fluorescence imaging (AFI) are mainly used as complementary tools to HD-WLE for the evaluation of large mucosal surfaces. In general, enhanced visualization is achieved by the application of nonspecific contrast agents such as methylene blue (i.e., for CE) or the use of specifically selected wavelengths (i.e., for NBI and AFI). More recently, optical molecular imaging has been translated into the clinic, using targeted molecular tracers that are labeled with fluorescent dyes as optical reporters. This molecular-based approach may be of added value for early lesion detection, by visualization of specific cellular and biological characteristics.

In parallel, there also is an increasing interest in cross-sectional imaging techniques that facilitate virtual histology, such as optical coherence tomography (OCT), confocal laser endomicroscopy (CLE) or endocytoscopy (EC). Obtaining *in vivo* histological information might enable real-time optical biopsies, thus contributing to faster diagnosis and potentially guiding interventions. However, the majority of these techniques have not yet been established as daily endoscopic imaging tools, nor incorporated into standard clinical endoscopes.

All of the above-mentioned endoscopic imaging enhancement techniques have specific advantages and disadvantages. In this article, we aim to provide an overview of the most recent developments and current clinical status of different endoscopic imaging enhancement techniques, that may be used to overcome current clinical challenges in mucosal lesion detection.

WIDE-FIELD IMAGING TECHNIQUES

Although high-definition white light endoscopy (HD-WLE) has significantly improved mucosal lesion identification over the past decades, there still is a significant miss-rate for detection of gastrointestinal neoplasia. This is particularly the case for small and flat lesions within the colon (Heresbach et al., 2008; Kaminski et al., 2010; van Rijn et al., 2006), or lesions with minimal morphological changes such as dysplasia in the esophagus and stomach (Sharma et al., 2013). To improve lesion identification, characterization, and diagnosis during endoscopy, numerous wide-field imaging techniques have been developed.

Chromoendoscopy

Chromoendoscopy (CE) is a diagnostic method that involves the topical application of stains or dyes onto the mucosal surface to improve lesion visualization and characterization during HD-WLE. After topical application using a spraying catheter that can be inserted through the working channel of a clinical video-endoscope, features of abnormal mucosal lesions can be inspected in more detail and vascular structures may become more prominent. A broad variety of stains or dyes are available for use in CE, which can be roughly classified into three groups: absorptive, reactive, and contrast dyes. Absorptive dyes such as methylene blue, lugol's solution, crystal violet or toluidine blue are absorbed across specific epithelial cell membranes. Reactive dyes such as congo red or phenol red change color based on chemical reactions within tissues that occur due to specific cellular constituents. Contrast dyes like indigo carmine highlight mucosal irregularities by pooling in the mucosal crevices (Fennerty, 1999).

CE is considered to be of added value for detection of dysplasia during surveillance endoscopies of patients with inflammatory bowel disease (IBD) (Choi et al., 2015; Kiesslich and Neurath, 2004; Marion et al., 2008; Matsumoto et al., 2003; Rutter et al., 2004). This population is at risk for developing colorectal cancer (CRC) by chronic inflammation of their intestines (Beaugerie and Itzkowitz, 2015). A recent prospective multicenter cohort study demonstrated an increased detection rate of 68.5% for all lesions and 57.4% for dysplastic lesions following CE-targeted biopsies in 350 patients (Carballal et al., 2018). However, there are also studies showing the contrary. For example, a retrospective multicenter study describing the use of CE in IBD patients over a period of 14 years showed no additional benefit for the detection of colorectal polyps (Mooiweer et al., 2015). Limitations in presented clinical trials such as small sample sizes, the use of CE only by expert endoscopists and an overestimation of dysplasia detection because of a second-look effect make it difficult to determine the added value of CE in current clinical practice. Moreover, CE does not

remove the need for random biopsies, as random biopsies performed in unsuspecting appearing mucosa of IBD patients still increases the neoplasia detection rate with 15% (Moussata et al., 2018).

The role of CE in non-IBD patients undergoing a colonoscopy for CRC screening also remains debatable. A recent Cochrane review on seven prospective randomized trials (2727 patients) showed that CE improved colorectal polyp detection, particularly for diminutive flat lesions (Brown et al., 2016). The largest randomized, prospective multicenter trial from this review with 1008 patients at risk for CRC showed that compared to HD-WLE, chromoendoscopy enhanced the overall detection of adenomas (0.95 vs. 0.66 per patient), flat adenomas (0.56 vs. 0.28 per patient), and serrated lesions (1.19 vs. 0.49 per patient) (Pohl et al., 2011). However, the quality of evidence was low because of methodological limitations and conservative assumptions that were made. Other potential reasons for variation may have been the different indications for colonoscopy.

In upper GI, CE does not seem to provide an additional value for the detection of mucosal abnormalities. For instance, indigo carmine CE has been investigated in BE, but specificity remains poor as mucosal changes that are associated with low-grade dysplasia (LGD) can hardly be distinguished from nondysplastic areas and neoplastic lesions appear the same as inflammation (Dawsey et al., 1998; Freitag et al., 1999; Yoshida et al., 2009). In gastric cancer, indigo carmine-based CE is only widely used in Japan to determine the resection margin status for endoscopic removal of gastric cancers, although there are several studies in which CE failed to delineate the complete resection margin (Nonaka et al., 2012; Yao et al., 2013).

In general, interpretation of observed CE staining patterns can be challenging and requires training, which is demonstrated by the significant interobserver variation in interpretation that was seen between several endoscopists who have participated in the studies described above. Moreover, dye spraying requires additional endoscopy time, which might be a hurdle in busy endoscopy clinics and CE is not feasible if bowel preparation is inadequate.

In summary, CE seems to be of added value for detection of neoplastic lesions in IBD. However, CE has not yet been established as a standard clinical endoscopic procedure for the detection of mucosal neoplasia in other patient categories for detection of neoplastic lesions in the GI tract.

Virtual Chromoendoscopy

Several endoscope manufactures have developed postprocessing software-based applications that adjust the wavelength ranges of reflected light in order to change endoscopic images in real-time, such as Pentax (i-scan), Fujinon (Flexible spectral Imaging Color Enhancement, FICE) or Karl Storz (STORZ Professional Image Enhancement System, SPIES). All three applications are relatively new in the field of gastroenterology. In contrast to NBI, different optical filter sets can be used, according to the preference of the endoscopist. As each wavelength corresponds to specific (sub)mucosal tissue characteristics, different effects on tissues, vasculature and mucosal surfaces can be highlighted (Rey et al., 2014).

Virtual chromoendoscopy in general consists of three digital modes: surface enhancement, contrast enhancement, and tone enhancement. The surface enhancement mode is used for better recognition of edges, through enhanced light-dark contrast by measuring the luminance intensity per pixel. Optimizing light-dark contrast ensures surface irregularities to become more prominent. With contrast enhancement, blue color is added to dark areas, thus also changing the way surface irregularities can be inspected. Tone enhancement is based on an algorithm that alters color frequencies of each red-green-blue component. By recombining these components, a new color image is reproduced. The endoscopist can switch between different modes by pushing a button on the endoscope. All three modes are arranged in series, which makes it possible to use multiple modes at the same time (Kodashima and Fujishiro, 2010).

I-scan is a novel technology developed by Pentax that enables the visualization of large surfaces due to its easy applicability (Figure 1). I-scan images are much brighter compared to the for example relatively dark NBI-images. A meta-analysis of 925 patients and 2312 colorectal polyps showed that i-scan highly accurately detects colonic polyps, and with a sensitivity and specificity to real-time predict polyp histology of 90.4% and 90.9% respectively (Guo et al., 2015). However, a high heterogeneity was observed in all studies, which is probably caused by different classification systems that were used. A more recent study showed that i-scan outperformed HD-WLE for the detection of diminutive small polyps in patients who underwent a colonoscopy for the first time (n=449 patients, $P=0.035$) (Shan et al., 2017). In contrast, the use of i-scan does not seem to contribute to the detection of dysplasia in BE (Lipman et al., 2017; Verna et al., 2014). For daily clinical practice it is difficult to advice the added value of the I-scan technology, as various i-scan modes have been used in the above-mentioned studies and a general classification system has not yet been established to decrease interobserver variation.

Figure 1. *i*-Scan images (Pentax) of a hyperplastic colon polyp (A), adenomatous colon polyp (B), and minimal change erosive esophagitis (C) that are acquired with surface and tone enhancement. Figure reprinted from East, J.E., Vleugels, J.L., Roelandt, P. et al. (2016). Advanced endoscopic imaging: European Society of Gastrointestinal Endoscopy (ESGE) technology review. *Endoscopy* 48, 1029–1045, with permission of Georg Thieme Verlag KG. © Georg Thieme Verlag KG.

Figure 2. Flexible spectral imaging color enhancement (FICE) images (Fujinon) of a hyperplastic colon polyp (A), adenomatous colon polyp (B), and squamous esophageal neoplasia (C) acquired with preset wavelengths: red 520 nm, gain 2; blue 405 nm, gain 3.

Figure reprinted from East, J.E., Vleugels, J.L., Roelandt, P., et al. (2016). Advanced endoscopic imaging: European Society of Gastrointestinal Endoscopy (ESGE) technology review. *Endoscopy* 48, 1029–1045, with permission of Georg Thieme Verlag KG. © Georg Thieme Verlag KG.

Flexible spectral imaging color enhancement (FICE) was developed by Fujinon and is being applied for the enhanced detection of mucosal lesions of the upper and lower gastrointestinal tract (Figure 2). To the best of our knowledge, this technique has only been used by expert endoscopists. In the first randomized, prospective multicenter study on 871 patients that were referred for screening and diagnostic colonoscopies, no significantly increase in adenoma detection was observed (Pohl et al., 2009). Other small studies in patients with ulcerative colitis and small-bowel capsule endoscopy did not show any benefits either (Cassinotti et al., 2018; Yung et al., 2017). There are also studies that demonstrate an improved detection of dysplastic lesions in BE patients, patients with early stage esophageal squamous cell carcinoma and gastric cancer (Camus et al., 2012; Li et al., 2014; Mouri et al., 2009). Comparable to i-scan, the improvement of definitions and a common classification system may optimize the added value of FICE.

The STORZ Professional Image Enhancement System (SPIES, Karl Storz) also provides several image enhancement modalities, but to the best of our knowledge, SPIES has not yet been investigated in clinical trials in the gastrointestinal tract.

Narrow Band Imaging

Narrow band imaging (NBI) is a type of virtual chromoendoscopy that is based on the use of a narrowed bandwidth of light to illuminate tissue of interest. Two optical filters that correspond to the hemoglobin absorption peaks of 440–460 nm (blue) and 540–560 nm (green) are placed in the light source unit of an endoscope. This enables the enhanced visualization of mucosal (micro)vasculature patterns, which are known to be altered in neoplastic tissue (Figure 3) (Boerwinkel et al., 2014; Gono, 2015). Neoplastic lesions usually appear darker compared to surrounding mucosa, allowing a real-time optical differentiation between neoplastic and nonneoplastic mucosa by NBI (McGill et al., 2013).

The role of NBI for the real-time detection of colorectal polyps is controversial. There is one randomized controlled trial demonstrating a trend toward an improved detection

for serrated colorectal lesions proximal of the sigmoid with NBI compared to HD-WLE, although the detection of conventional adenomas in this trial was similar for both modalities (Rex et al., 2016). On the other hand, a Cochrane review of 8 randomized trials with 3673 participants showed no statistically significant difference between standard HD-WLE and NBI for the detection of patients with colorectal polyps (Nagorni et al., 2012), nor did a meta-analysis of prospective, randomized trials of in total 293 patients (Dinesen et al., 2012).

Figure 3. Narrow-band imaging (NBI) of a neoplastic lesion in a patient with BE, demonstrating the enhanced visualization of the mucosal (micro)vasculature pattern of the neoplasia in an overview image (B), detailed image (D) and near-focus (F) image compared to high-definition white-light endoscopy in the left column (A, C, E). The border of the vascular and mucosal irregularities based on NBI is depicted in red and the extension of the lesion based on the mucosal relief is depicted in blue.

© Figure reprinted from Boerwinkel, D.F., Swager, A., Curvers, W.L., Bergman, J.J. (2014). The clinical consequences of advanced imaging techniques in Barrett's esophagus. *Gastroenterology* 146, 622–629, with permission from Elsevier.

There is promising data on the use of NBI for surveillance endoscopy in patients with BE. NBI enabled the detection of a higher proportion of dysplasia in 123 BE patients (30% vs. 21%) (Sharma et al., 2013). Moreover, NBI required fewer biopsies to be taken per patient (3.6 vs. 7.6; $P < 0.0001$), meaning that NBI may reduce the number of random four-quadrant biopsies at set intervals along the BE segment (Singh et al., 2011). A meta-analysis including 502 patients with BE showed that NBI had a sensitivity and specificity of 91% and 95% respectively for identifying high-grade dysplasia (HGD) and a sensitivity and specificity of 91% and 85% respectively for detecting specialized intestinal metaplasia (Song et al., 2015). Although recent technological advances have been made like an optical zoom for improved visualization of mucosal patterns, NBI is not yet implied in the guidelines for standard BE surveillance (Sturm and Wang, 2015). However, it is already being used in many Barrett expert centers and the American College of Gastroenterology-guideline does recommend to use NBI after endoscopic treatment to detect mucosal abnormalities in BE patients.

NBI can also be of added value for the accurate detection of early gastric cancer. A multicenter prospective study with 1351 patients has demonstrated a better detection of depressed lesions that are smaller than 10 mm and a better real-time diagnostic accuracy for gastric cancer detection by NBI compared to WLE (Ezoe et al., 2011). To further improve the endoscopist capability for diagnosing early gastric cancers, e-learning systems have been developed that are based on a vessel plus surface classification system (Nakanishi et al., 2017; Yao et al., 2016).

The main advantage of NBI is that it can easily be educated and that validated performance assessments are available for the nonexpert endoscopist. Moreover, it does not require prolongation or adjusted endoscopy procedures and lesions can be detected without the need to administer dyes. However, more longitudinal studies with non-expert and expert endoscopists are required to elucidate the precise role of NBI for different indications.

Auto Fluorescence Imaging

Auto fluorescence imaging (AFI) makes use of blue light (390–470 nm) to illuminate mucosal surfaces for the detection of tissue autofluorescence that is emitted by endogenous molecules, such as collagen, flavins or porphyrins. The principle of fluorescence is based on excitation of a fluorophore by light of a specific wavelength, after which light is emitted with a longer wavelength. In AFI, tissue of interest is excited with a blue (390–470 nm) and a green light (540–560 nm). Subsequently, the blue light is filtered out so that only the tissue autofluorescence and reflected green light is detected. This creates an autofluorescence image, in which dysplasia is shown as purple and normal mucosa as green (Figure 4) (East et al., 2016). The degree of tissue autofluorescence emission depends on differences in tissue features. Changes in mucosal thickness and mucosal blood flow influence the autofluorescence signals (Falk, 2009; Song et al., 2011).

Although AFI enables the differentiation between dysplastic and non-dysplastic tissue, it does not seem to significantly reduce the adenoma miss-rate during colonoscopy

(Kuiper et al., 2011). However, it might be of added value during surveillance of neoplasia in ulcerative colitis patients, although the limited patient number in this study ($n=25$) necessitates further research to determine the diagnostic accuracy (van den Broek et al., 2008). AFI is not considered to improve the detection of early neoplasia in BE patients in general practice (Curvers et al., 2011).

AFI, NBI, and HD-WLE can also be combined, that is, Endoscopic TriModal Imaging (ETMI). So far, ETMI has mainly been studied in an academic setting, when compared to HD-WLE for the detection of colorectal lesions. There is one study describing the application of ETMI in a nonacademic setting, in which the adenoma detection rate was not improved (Kuiper et al., 2011).

Figure 4. Autofluorescence imaging (AFI) of three neoplastic lesions in patients with Barrett's esophagus that are difficult to detect using white-light endoscopy (A, C, E), but appear more clearly when using autofluorescence imaging (B, D, F). Lesions that are located at the gastric folds can be more challenging to detect by AFI (F).

© Figure reprinted from Boerwinkel, D.F., Swager, A., Curvers, W.L., Bergman, J.J. (2014). The clinical consequences of advanced imaging techniques in Barrett's esophagus. *Gastroenterology* 146, 622–629, with permission from Elsevier.

Although AFI may enhance the detection of dysplastic lesions for some indications, it has not yet been validated as standard endoscopic technique in larger studies. Compared to HD-WLE, this technique has a high percentage of false-positive lesions. Another disadvantage is the reduced endoscopic flexibility, where rapid movement of the endoscope lead to degradation of image quality. Technological developments, such as image resolution, noise reduction, and color contrast are needed to further improve the accuracy of AFI compared to HD-WLE (Song et al., 2011).

Optical Molecular Imaging

As abovementioned, imaging enhancement techniques such as chromoendoscopy, NBI and AFI are based on the enhanced visualization of morphological characteristics of mucosal lesions. Molecular imaging is an emerging technique that provides contrast through application of for example fluorescently labeled imaging agents that specifically target molecular characteristics of cells, such as receptors or proteins that become overexpressed in dysplastic lesions. Application of targeted imaging agents might therefore improve imaging specificity (Pierce et al., 2008). The real-time visualization of biological characteristics of cells in addition to HD-WLE enables the rapid evaluation of mucosal surfaces, without necessarily the need for visible morphological changes. Therefore, premalignant lesions could potentially be detected at an earlier stage, which has raised increasing interest in the endoscopic application of molecular imaging.

Optimal target selection is essential for the accurate detection of cancer and precancerous lesions. Cancer development is thought to be a multistep process caused by genetic alterations, resulting in the upregulation of a broad variety of biomarkers, such as upregulated receptors. After selection of potential biomarkers, the further validation of a target by for example immunohistochemistry, *in vitro* or *in vivo* experiments remains essential before clinical translation.

Molecular fluorescence imaging is a method that can be applied to visualize biomarker expression levels *in vivo*. There are numerous fluorescent dyes available for conjugation to a “carrier” molecule. Fluorescent dyes that emit light in the near-infrared (NIR) light spectrum (700–900 nm) have several advantages over dyes that emit light in the visible light spectrum (380–700 nm), such as lower tissue absorption and reflectance properties and minimal autofluorescence. As a result, NIR light can penetrate deeper into tissue, up to ± 1 cm (Weissleder and Ntziachristos, 2002). The majority of clinical studies that have been performed in endoscopy so far have therefore favored the use of NIR fluorescent dyes over fluorescent dyes that emit light in the visible light spectrum.

There are also various types of targeting moieties that can be used for conjugation to fluorescent dyes, such as lectins, small molecules, peptides, antibodies or activatable molecules, that is, molecules that become activated after enzymatic cleavage. Smaller molecular sizes generally result in faster pharmacokinetic properties. This provides a rapid tissue distribution and relatively short half-life, but also minimizes your optimal imaging window and potentially uptake in the target area. Moreover, conjugating a small molecule to a fluorescent dye can be more challenging, as small structural changes might highly impact pharmacokinetic properties and binding affinity (Zhang et al., 2017).

Targeted fluorescent agents can be administered intravenously (IV) or topically by spraying onto the mucosal surface. A different method of delivery can be used per indication. The time between IV administration and endoscopy differs per fluorescent agent, depending on its pharmacokinetics and pharmacodynamics. IV administration results in a homogenous distribution throughout the body, penetrating all layers of the gastrointestinal wall. However, this potentially also increases background fluorescence derived from the remaining circulating fluorescent agent. Topical application provides fast contrast within minutes, with a substantial lower risk of adverse events and toxicity, but can be challenging due to for example inhomogeneous distribution, required passage of mucus barriers and nonspecific binding. Although anti-mucolytic substances like acetylcysteine or permeation enhancers like ethanol can be used to improve permeability, this remains challenging. Moreover, as topical application prohibits from spraying larger and less cleaned surfaces such as the colon, topical application may be more suitable for diagnostic upper gastroscopic applications (Goetz et al., 2013; Pierce et al., 2008).

To visualize fluorescence with a specific wavelength, custom developed (NIR) fluorescence endoscopy systems are being used in clinical trials for visualization of fluorescence *in vivo*. These systems can either be integrated in current HD video-endoscopes or fiber-based. The main limiting factor for developing integrated (NIR) fluorescence endoscopes is that the small charge-coupled device chip that is embedded in the tip of the endoscope cannot yet provide sufficient sensitivity for NIR fluorescence imaging. Therefore, coherent fiber-optic bundles that can be inserted through the working channel of a standard HD video-endoscope have been developed as an alternative, to allow simultaneous white-light and fluorescence imaging by use of different filter sets and a dichroic mirror, alongside standard HD-WLE (Garcia-Allende et al., 2014). Using such fluorescence camera systems combined with targeted fluorescent agents provides real-time high-resolution imaging of mucosal surfaces in a non-invasive manner, which has the potential to be used as a red-flag technique to identify dysplasia or cancer.

Clinical studies on fluorescence molecular endoscopy (FME)

A broad variety of fluorescent probes have been tested *ex vivo* in preclinical animal models with promising results. Over the past decade, this has led to the clinical translation of optical molecular imaging using targeted fluorescent probes both intravenously and topically for the endoscopic detection of (pre)malignant lesions in humans. So far, the first clinical studies have mainly focused on the detection of (pre)malignant lesions in patients with Barrett's esophagus, detection of colorectal polyps and evaluation of treatment response in IBD patients (Atreya et al., 2014; Burggraaf et al., 2015; Hartmans et al., 2018; Joshi et al., 2017; Nagengast et al., 2019).

Barrett's esophagus

In Barrett's esophagus (BE), there is a detection miss-rate for high-grade dysplasia (HGD) and early esophageal adenocarcinoma (EAC) of up to 25.3% (Visrodia et al., 2019). Therefore, novel imaging techniques are needed to improve the early detection of dysplasia

and esophageal adenocarcinoma in BE during surveillance endoscopy. Fluorescence molecular endoscopy (FME) has already been investigated in BE patients for early lesion detection using different fluorescent probes, such as ASY*-FITC (Joshi et al., 2017) or bevacizumab-800CW (Nagengast et al., 2019).

ASY*-FITC consists of the peptide ASY-NYDA, which was found to target dysplastic esophageal mucosa using phage display, that was labeled to fluorescein isothiocyanate (FITC, peak emission in the visible light spectrum at 519 nm). At first, this probe was used for detection of (pre)malignant lesions in BE patients using a probe-based confocal laser endomicroscopy (pCLE). HGD and EAC foci could be distinguished from normal squamous epithelium and BE with a ratio of 3.8, potentially identifying areas for biopsy or endoscopic therapy (Sturm et al., 2013). However, as pCLE does not provide wide-field imaging and only has limited penetration depth, this approach is not suitable for use as a red-flag imaging technique. Therefore, the same research group investigated the feasibility of FME using topical application of ASY*-FITC in BE patients. Fluorescence was visualized using an AFI endoscope with a modified filter set to match the FITC fluorescence spectrum. Joshi et al. showed that HGD and early EAC lesions could be detected in 50 Barrett's esophagus patients using this method, with a sensitivity and specificity of 76% and 94% respectively (Joshi et al., 2016). In this study, a postprocessing segmentation algorithm was used, which currently still prohibits real-time lesion identification during endoscopy.

Bevacizumab-800CW is a NIR fluorescent tracer that consists of the monoclonal antibody bevacizumab targeting the vascular endothelial growth factor-A (VEGF-A), which is labeled to the NIR fluorescent dye IRDye800CW (peak emission at 789 nm) (ter Weele et al., 2016). Using a fiber-based NIR fluorescence endoscopy system, bevacizumab-800CW was used for detection of dysplastic or early esophageal adenocarcinoma lesions in patients with Barrett's esophagus (Figure 5) (Nagengast et al., 2019). Following both IV and topical administration of bevacizumab-800CW, all visible lesions in the Barrett's segment could be detected real-time in 14 patients. Interestingly, the topical application of bevacizumab-800CW resulted in an overall increase in lesion detection of 33% compared to HD-WLE (Nagengast et al., 2019). To demonstrate the diagnostic accuracy of this technique in a larger patient cohort, a phase-II clinical trial is currently being initiated.

Colorectal polyps

Today, HD-WLE is the gold standard for detection of (pre)malignant lesions during colonoscopy. However, small, flat, and particularly right-sided adenomas are known to be difficult to detect with miss-rates of up to even 55% in Lynch syndrome patients (Stoffel et al., 2008; van Rijn et al., 2006). As with upper endoscopy, the feasibility of molecular imaging for this purpose has already been investigated using several different targeted fluorescent probes.

The first *in vivo* feasibility study using targeted fluorescence imaging for improved colorectal polyp detection has been performed already by Keller et al (2002). In this study, the topical application of a fluorescein-labeled monoclonal antibody targeting carcinoembryonic antigen was investigated in polypoid lesions that were previously

A

B

C

Figure 5. Fluorescence molecular endoscopy (FME) using the near-infrared fluorescent tracer bevacizumab-800CW targeting vascular endothelial growth factor A (VEGF-A). Panel A describes a schematic overview of two FME approaches: intravenous (1) and topical (2) application. Panel B shows representative examples of the detection of a flat, elevated and protruding lesion by FME after intravenous administration of bevacizumab-800CW (upper, middle, and bottom row respectively). High-definition white-light endoscopy (HW-WLE) images are displayed in the left column, an overlay of fluorescence with a white-light fiber image in the middle column, and the fluorescence image alone in the right column. Panel C shows representative examples of the detection of a carcinoma lesion that was missed by HD-WLE, a flat lesion and a protruding lesion by FME after topical application of bevacizumab-800CW (upper, middle, and bottom row respectively).

Figure is adapted and reproduced from Nagengast, W.B., Hartmans, E., Garcia-Allende, P.B. et al. (2019). Near-infrared fluorescence molecular endoscopy detects dysplastic oesophageal lesions using topical and systemic tracer of vascular endothelial growth factor A. Gut 68 (1), 7–10, with permission from BMJ Publishing Group Limited.

detected in the colon of 27 patients. Using a conventional fiberglass endoscope (Olympus) coupled to a single-reflex camera, 19/25 carcinomas and 3/8 adenomas could be visualized using fluorescence. Carcinoma lesions could not be seen using fluorescence in the presence of bleeding or mucosal ulceration. However, this study did not investigate the detection of additional polyps, as topical application onto the colon is not considered to be optimal due to its large surface.

Other studies have been performed to investigate the intravenous administration of targeted fluorescent probes during colonoscopy. Joshi et al. have demonstrated the potential of a fluorescently labeled peptide KCC*-FITC, targeting the V600E BRAF point mutation, for the detection of sessile serrated adenomas (SSAs) (Joshi et al., 2017). A multimodal video colonoscope (CF-Y0012, Olympus Medical Systems Corp) with two separate standard definition detectors (i.e., for fluorescence/reflectance and white-light images) was used. A total of 44 lesions in 38 patients that were initially identified using conventional HD-WLE, were subsequently administered topically with KCC*-FITC. Using a postprocessing algorithm, the authors found a sensitivity and specificity of 89% and 92% for discriminating SSAs from normal colon mucosa. Moreover, they observed a sensitivity and specificity of 84% and 91% for discriminating SSA from adenoma at a tumor-to-background ratio of 1.66. However, the use of a postprocessing algorithm prohibits real-time lesion identification.

Two other groups have demonstrated the feasibility of *in vivo* real-time application of targeted fluorescent tracers. Firstly, Burggraaf et al. described the development, safety and in human use of GE-137, a 26-amino acid cyclic peptide labeled to a Cy-5 derived dye that targets the membrane receptor c-Met (peak emission at 675 nm) (Burggraaf et al., 2015). C-Met is known to be overexpressed already early in the colorectal adenoma-carcinoma sequence. GE-137 was administered intravenously 3h before endoscopy and seemed to be well-tolerated and safe in healthy volunteers and patients at high risk of colorectal neoplasia (n=31). For fluorescence-guided colonoscopy, a custom image head and illumination module were mounted to the proximal end of a fiber colonoscope (Pentax, FC-38LV). Fifteen patients with a high risk of colorectal neoplasia were included. Twelve additional adenomas were detected using second-pass combined white-light and fluorescence inspection, of which nine lesions were only visible using fluorescence (Figure 6) (Burggraaf et al., 2015). However, also the majority of benign hyperplastic polyps showed increased levels of fluorescence. Although results seem promising, the use of a tracer in the visible light spectrum requires adaptations of current clinical endoscopes, as the red light of the excitation laser may be detected with the HD-WL endoscope and compromise white-light inspection.

Secondly, Hartmans et al. performed a dose-finding feasibility and safety study with the NIR fluorescent tracer bevacizumab-800CW targeting VEGF-A, for colorectal adenoma detection in 17 patients with familial adenomatous polyposis (Hartmans et al., 2018). Bevacizumab-800CW was administered three days prior to endoscopy. Real-time fluorescence imaging was performed using a fiber-probe that was inserted through the

working channel of the standard HD video-endoscope. There were no adverse events related to bevacizumab-800CW. All adenomas that were visualized with HD-WLE were also detected with fluorescence in all three dose cohorts (i.e., 4.5, 10, and 25 mg of bevacizumab-800CW). No false positive fluorescence signals were observed. The authors report that 25 mg of bevacizumab-800CW was the most optimal tracer dose to enable the real-time detection of small (<3 mm) adenomas. However, the IV administration of bevacizumab-800CW three days prior to endoscopy is unpractical for clinical translation of this tracer for polyp detection.

These three feasibility studies clearly indicate the feasibility of optical molecular imaging for adenoma detection during endoscopy of the lower gastrointestinal tract. However, larger diagnostic accuracy studies are needed to validate the reported sensitivity and specificity's in these feasibility trials. Moreover, implementation of fluorescence imaging techniques into standard HD video-endoscopes and further improvements in sensitivity, together with the development of novel specific fluorescent tracers with favorable pharmacokinetics can benefit clinical implementation.

Evaluation of treatment response

As optical molecular imaging can provide insight in biological processes at a cellular level, it might be of added value for the evaluation of therapeutic responses. Expensive treatment strategies such as antibodies for inflammatory bowel disease (IBD) or neoadjuvant chemoradiotherapy (nCRT) for locally advanced cancer patients are given, which may not be as effective for each patient individually and potentially cause side-effects. Therefore, there is a need to predict the therapeutic response in order to enable more patient-tailored treatment strategies.

Antibodies like adalimumab or infliximab block the tumor necrosis factor and can be highly effective for induction of remission in patients with moderate to severe Crohn's disease. However, $\pm 50\%$ of patients do not respond to adalimumab (Hanauer et al., 2006). As identification of the transmembrane Tumor Necrosis Factor (mTNF) might be predictive for treatment efficacy, Atreya et al. have labeled adalimumab to the fluorescent dye FITC and performed an *in vivo* fluorescence imaging study. Twenty-five patients with confirmed active Crohn's disease were included. Fluorescence was visualized after topical application of adalimumab-FITC using a Pentax Endomicroscopy System: a HD-WL video endoscope with an integrated confocal fluorescence microscope (see "Confocal Laser Endomicroscopy" section). Patients with high mTNF expression, determined as the number of fluorescent mTNF cells per confocal image, showed a clinical response to adalimumab therapy in 92% versus 15% in patients with low mTNF expression levels. A follow-up endoscopy with a median of 17 months was performed in patients from the high mTNF group, which showed mucosal healing in 73% of patients with significantly reduced inflammation scores (Atreya et al., 2014). Although the field of view of a confocal laserndomicroscopy system is limited and therefore the number of fluorescent mTNF cells

Figure 6. Fluorescence molecular endoscopy (FME) using the fluorescent tracer EMI-137 targeting c-Met. A white-light image is depicted followed by a fluorescent image of a pedunculated tubular adenoma (A), sub-pedunculated tubulovillous adenoma (B), sessile serrated polyp (C), flat elevated tubular adenoma (D), and two flat tubular adenomas (E and F). The relationships between the diagnosis and the level of fluorescence (G), the level of fluorescence and c-Met expression (H) and the diagnosis and level of c-Met expression (I) are displayed in graphs.

© Figure reprinted from Burggraaf, J., Kamerling, I.M.C., Gordon, P.B. et al. (2015). Detection of colorectal polyps in humans using an intravenously administered fluorescent peptide targeted against c-Met. *Nature Medicine* 21, 955–961, with permission by Springer Nature.

that can be detected may vary per image, which leads to a variation in results, the results seem very promising. A larger study needs to be performed to further characterize the performance of adalimumab-FITC in Crohn's disease.

For prediction of treatment response after neoadjuvant chemoradiotherapy, the aim is slightly different. Approximately 26.5% and 15%–27% of locally advanced esophageal and rectal cancer patients have a pathologically complete response (pCR) after nCRT, meaning no residual tumor is present during histopathological analysis of the resected specimens (Duan et al., 2014; Maas et al., 2010; Park et al., 2012). However, in current clinical practice, independent of the response to nCRT, all patients receive extensive surgical procedures. For locally advanced rectal cancer (LARC) patients, HD-WLE and magnetic resonance imaging (MRI)-scans are already being applied in clinical practice to assess therapeutic response rates in order to select patients for an active wait-and-see approach (Martens et al., 2016). However, HD-WLE only provides superficial morphological information and differentiation between tumor and fibrosis is often difficult on MRI scans. Therefore, optical molecular imaging might assist in evaluating treatment response by providing insight at a cellular level. This approach is currently being investigated using the NIR fluorescent tracer bevacizumab-800CW for both locally advanced rectal and esophageal cancer patients (NCT01972373, NCT03558724). Fluorescence levels after nCRT are quantified *in vivo* at the site of the tumor by correcting for optical properties of the tissue (i.e., scattering and absorption) using multi-diameter single-fiber reflectance, single fiber fluorescence spectroscopy. Future results will demonstrate if a fluorescence cut-off value can be determined to select patients with a pCR after nCRT.

CROSS-SECTIONAL IMAGING TECHNIQUES

Optical Coherence Tomography

Optical coherence tomography (OCT) typically makes use of a NIR low-coherence laser for imaging of tissue morphology. The delay in time it takes for the light to scatter or reflect back enables the real-time reconstruction of sub-micrometer resolution, cross-sectional images of tissue structures. In a way the mechanism of action of OCT is thus comparable to ultrasound, but instead of an acoustic signal, NIR light is used. Although the axial resolution of OCT is relatively high with 1–10 μm , the trade-off is a limited penetration depth of up to 1–2 mm due to optical attenuation from tissue optical properties (Fujimoto et al., 2000; Thompson et al., 2017).

OCT was initially developed for ophthalmologic indications such as imaging of the retina, but improvements over the past decades have enabled a fiber-based approach to allow extension into the field of gastroenterology for different indications. Up to date, only prototype OCT systems are available, that use a catheter-based probe that can be inserted through the working channel of a clinical HD video-endoscope. The first prospective and still the largest study performed so far dates back to 2001, in which the sensitivity and specificity of OCT for diagnosing specialized intestinal metaplasia was investigated in 121 patients receiving upper endoscopy. The authors established three criteria to diagnose BE: the lack of normal morphology, the presence of submucosal glands and inhomogeneous

contrast. When applying two of these criteria, a sensitivity and specificity of 97% and 92% respectively was found compared to histology (Poneros et al., 2001). The same results were obtained in a comparable study in 2007, where specialized intestinal metaplasia could be prospectively diagnosed with a sensitivity and specificity of 97% and 92% (Evans et al., 2007). However, the detection of dysplasia in patients with BE still remains challenging. In 33 BE patients, the sensitivity and specificity for detection of dysplasia (low-grade, high-grade and intramucosal cancer) were 68% and 82%, decreasing as the grade of dysplasia progressed (Isenberg et al., 2005). Recent data however is lacking.

More recently, OCT has been compared to endoscopic ultrasound for its accuracy to preoperatively stage 123 patients with superficial esophageal squamous cell carcinomas limited to the epithelium or lamina propria mucosa. OCT showed an accuracy for determining whether the carcinoma was limited to the epithelium or lamina propria mucosa of 94.6% versus 80.6% for endoscopic ultrasound, with better interobserver agreement (Hatta et al., 2012). Further development of the technique has enabled 3D-OCT, which has been investigated for the detection of buried glands before and after radiofrequency ablation in 27 patients with BE. 3D-OCT detected buried glands in 63% of patients after radiofrequency ablation, highlighting another potential application of OCT (Zhou et al., 2012).

Application of OCT in the colon has been studied less frequently, so there is limited clinical data available, especially for the detection of dysplasia. Previously, criteria have been described to distinguish colorectal adenomas from hyperplastic polyps based on the results of 24 patients in which 44 colorectal polyps were imaged using OCT and subsequently removed (Pfau et al., 2019). Moreover, it has been described that OCT has a sensitivity and specificity of 90.0% and 83.3% respectively for the detection of transmural inflammation, potentially aiding in the diagnosis of inflammatory bowel disease (Shen et al., 2004). Further feasibility studies are required to determine the clinical benefit of OCT in the lower gastrointestinal tract.

In general, despite these promising results the main limitations of OCT are the limited penetration depth of 1–3 mm, low contrast images because of lack of internal contrast and the interobserver variability regarding interpretation of images and correlation to histopathology.

Volumetric Laser Endomicroscopy

Volumetric laser endomicroscopy (VLE) is a second-generation OCT system in which a balloon-based imaging probe is used to collect volumetric scans with a similar penetration depth and an axial resolution of 7 mm. The balloon is 6 cm long and enables centering of the fiber-probe during scanning of the esophagus, to optimize imaging results.

The safety and feasibility of VLE was demonstrated in 100 patients with BE, showing a successful VLE completion rate of 87% of patients (Wolfsen et al., 2015). The VLE system has been adjusted to enable laser cautery-marking, which can be used *in vivo* to highlight areas of interest for biopsy. The safety and feasibility of VLE-guided biopsy using this approach was demonstrated in 22 patients with BE. Using VLE with laser cautery

marking, lesions could be diagnosed correctly with an accuracy of 93% and 100% for postprocessed VLE images (Suter et al., 2014). Currently, a United States multi-center registry prospectively collects data when VLE is being used for BE management to determine the clinical benefit of this technique.

Tethered Capsule Endomicroscopy

Tethered capsule endomicroscopy (TCE), also known as tethered capsule OCT, uses a different form of OCT called optical frequency domain imaging (OFDI). Cross-sectional microscopic images are collected through a capsule that can be swallowed by the patient, enabling three-dimensional, morphological imaging of the (upper) gastrointestinal tract (Figure 7) (Gora et al., 2013). The capsule itself has a length of 24.8 mm and a diameter of 12.8 mm and is connected to a flexible tether, by which it can be extracted after imaging and its position can be controlled. The cross-sectional microscopic images of the esophageal wall are acquired after swallowing in ± 60 seconds at a 30 mm lateral and 7 mm axial resolution.

TCE is much less invasive compared to standard HD-WLE and can be applied in non-sedated patients. Gora et al. have demonstrated the technical feasibility in a total of 26 patients (11 healthy volunteers, 2 with gastroesophageal reflux, 10 with BE and 3 for the indication of eosinophilic esophagitis), of which 24 were able to swallow the capsule and imaging could be performed. Next, the same group showed that the TCE procedure is feasible in the primary care setting for screening purposes, showing a swallowing rate of 85% out of 20 patients, without sedation or any subject preparation (Gora et al., 2016). As more clinical data becomes available, the technique is further optimized by development of a fully automated segmentation algorithm and better characterization of the esophageal wall. Very recent, the safety and feasibility of TCE was again demonstrated for imaging of the esophagus, stomach and 20 cm of duodenum in 34 patients (Gora et al., 2018). This highlights the possibility for TCE to be used as a screening tool for BE patients. However, limited clinical data on diagnosing dysplasia in BE is available, so further studies are needed to demonstrate the clinical efficacy.

Confocal Laser Endomicroscopy

Confocal laser endomicroscopy (CLE) is a technique that is developed to enable imaging of the mucosa at a cellular and subcellular level, with a high magnification and resolution that is similar to conventional histological examination. In short, a low-power laser is used for tissue illumination, which is focused at a specific depth. Subsequently, reflected light is detected through a pinhole and refocused onto the detection system by the same lens. As a result, scattered or reflected light that originates below or above the tissue of interest is not detected, thus increasing spatial resolution (Chauhan et al., 2014).

CLE is mainly used in a probe-based approach (pCLE, Cellvizio, Mauna Kea Technologies, Paris, France), although some dedicated endoscopes have been developed with an integrated CLE fiber in the distal tip of the endoscope. CLE imaging can either be based on the detection of light that is reflected back, or on the detection of light that is

derived from excitation of an exogenous fluorescent contrast agent. Mainly non-targeted fluorescent contrast agents like fluorescein are administered intravenously to provide more contrast and therefore a resolution, generating images comparable to histopathology.

Figure 7. Tethered capsule endomicroscopy (TCE). The TCE-device (A) is swallowed through the esophagus and then pulled back for evaluation of the esophageal mucosa using the optical coherence tomography (OCT) technology (B). Panel C shows a structure that most likely corresponds to a blood vessel (red arrow). Panel D shows the different mucosal layers of the esophagus: epithelium (e), lamina propria (l), muscularis mucosa (m), submucosa (s), muscularis propria (mp), and adventitia (a). Panel E shows thickened squamous epithelium (green line) and high reflectance from the surface (blue dashed line) of a physiologic transformation zone that bridges stratified squamous mucosa shown in panel D. Panel F shows gastric cardia mucosa with a pit pattern (blue arrows).

© Figure reprinted Gora, M.J., Quénéhervé, L., Carruth, R.W. et al. (2018). Tethered capsule endomicroscopy for microscopic imaging of the esophagus, stomach, and duodenum without sedation in humans (with video). *Gastrointestinal Endoscopy* 88, 830–840, with permission from Elsevier.

The main advantage of CLE is that real-time “optical biopsies” can be obtained of any endoluminal surface, enabling targeted biopsies, and potentially decreasing conventional

biopsy numbers. To date, the technique is mainly being applied for the detection of dysplasia and early carcinomas in Barrett's esophagus or Crohn's disease, and for polyp classification.

Endocytoscopy

Endocytoscopy (EC) enables the real-time observation of cell nuclei with a 360–450x magnification through a contact light microscopy system that can be integrated into the distal tip of HD video-endoscope. Typically, a mucosal staining using a dye like methylene blue is applied before performing EC. As with CLE, interpretation of CE-images requires training for endoscopist on pathological characteristics of mucosal abnormalities.

Few clinical studies have been performed so far using EC. Initially, a probe-based EC-system was used following methylene blue application in the esophagus (Inoue et al., 2006; Kumagai et al., 2004, 2009). More recently, EC has been investigated for the detection of colorectal neoplasia using an integrated endocytoscope (Olympus Corp.) (Cipolletta et al., 2009; Kudo et al., 2011; Mori et al., 2013). EC proved to be noninferior to standard biopsy for the diagnosis of colorectal neoplasia, with a diagnostic accuracy of 94.1% versus 96.0% respectively (Mori et al., 2013). This suggests EC could be an alternative to standard biopsy, as it has the potential to provide a diagnosis during endoscopy. The feasibility of EC has also been investigated in a small feasibility study in patients with gastric cancer, showing a promising diagnostic accuracy as well (Kaise et al., 2014).

To improve endoscopist training, an automated system for endocytoscopic diagnosis has been developed, to optimize the accuracy of EC (Mori et al., 2016). Moreover, accuracy was further improved by a computer-aided diagnostic system for EC, which was validated for colorectal lesions (Mori et al., 2015). The main limitation of EC is that it only provides information on nuclear morphology and that topical application of staining dyes is required, which prohibits the visualization of large mucosal surfaces and can result in poor staining due to mucus or plaque. Although EC seems to be a clinically feasible technique, data on the diagnostic accuracy still needs to be determined in future studies.

CONCLUSION

Improvements in wide-field imaging modalities such as optical chromoendoscopy, NBI and AFI have greatly influenced the field of gastroenterology, although many novel mucosal imaging enhancement techniques have been developed in recent years that show great potential as well. Virtual chromoendoscopy techniques like i-Scan, FICE or SPIES enable better contrast and a higher image resolution, but they still mainly rely on the presence of morphological tissue characteristics for the detection and characterization of lesions. To further improve mucosal lesion detection, “red-flag” imaging techniques may be of added value that provide contrast based on the molecular characteristics of a lesion. Although techniques like optical molecular imaging (fluorescence molecular endoscopy) or optical coherence tomography show great potential in early-phase clinical studies, their diagnostic accuracy and efficacy should be demonstrated in sufficiently powered randomized

clinical trials. In addition, cross-sectional imaging techniques such as optical coherence tomography, confocal laser endomicroscopy or endocytoscopy aim to facilitate *in vivo* histology by providing “optical biopsies,” thus potentially contributing to faster diagnosis and potentially guiding interventions. With the development of so many novel imaging techniques, a collaborative and multidisciplinary approach is essential to speed up clinical translation and perform sufficiently powered randomized clinical trials.

FUTURE PERSPECTIVES

The development of novel imaging techniques provides an enormous amount of visual data and information. It is therefore crucial to assure that the endoscopist is sufficiently trained on how to interpret these data. Interactive web-based training modules might assist in training and improve mucosal lesion detection and staging. Besides training, there may be a role for standardized computer-aided detection algorithms to optimize image processing and interpretation. Computer-aided diagnosis may very well be used in the future to improve the accuracy of the endoscopist.

REFERENCES

- Atreya R, Neumann H, Neufert C, et al. (2014) In vivo imaging using fluorescent antibodies to tumor necrosis factor predicts therapeutic response in Crohn's disease. *Nature Medicine* 20: 313–321.
- Barclay RL, Vicari JJ, Doughty AS, Johanson JF, and Greenlaw RL (2006) Colonoscopic withdrawal times and adenoma detection during screening colonoscopy. *The New England Journal of Medicine* 355: 2533–2541.
- Beaugerie L and Itzkowitz SH (2015) Cancers complicating inflammatory bowel disease. *The New England Journal of Medicine* 373: 195.
- Boerwinkel DF, Swager A, Curvers WL, and Bergman JJ (2014) The clinical consequences of advanced imaging techniques in Barrett's esophagus. *Gastroenterology* 146: 622–629.
- Brown SR, Baraza W, Din S, and Riley S (2016) Chromoscopy versus conventional endoscopy for the detection of polyps in the colon and rectum. *The Cochrane Database of Systematic Reviews* 4: CD006439.
- Burggraaf J, Kamerling IMC, Gordon PB, et al. (2015) Detection of colorectal polyps in humans using an intravenously administered fluorescent peptide targeted against c-Met. *Nature Medicine* 21: 955–961.
- Camus M, Coriat R, Leblanc S, et al. (2012) Helpfulness of the combination of acetic acid and FICE in the detection of Barrett's epithelium and Barrett's associated neoplasias. *World Journal of Gastroenterology* 18: 1921–1925.
- Carballal S, Maisterra S, López-Serrano A, et al. (2018) Real-life chromoendoscopy for neoplasia detection and characterisation in long-standing IBD. *Gut* 67: 70–78.
- Cassinotti A, Buffoli F, Fociani P, et al. (2018) Virtual chromoendoscopy with FICE for the classification of polypoid and nonpolypoid raised lesions in ulcerative colitis. *Journal of Clinical Gastroenterology* 2: 1–4.
- Chauhan SS, Abu Dayyeh BK, Bhat YM, et al. (2014) Confocal laser endomicroscopy. *Gastrointestinal Endoscopy* 80: 928–938.
- Choi HR, Rutter MD, Askari A, et al. (2015) Forty-year analysis of colonoscopic surveillance program for neoplasia in ulcerative colitis: An updated overview. *The American Journal of Gastroenterology* 110: 1022–1034.
- Cipolletta L, Bianco M, Rotondano G, et al. (2009) Endocytoscopy can identify dysplasia in aberrant crypt foci of the colorectum: A prospective in vivo study. *Endoscopy* 41: 129–132.
- Curvers WL, van Vilsteren FG, Baak LC, et al. (2011) Endoscopic trimodal imaging versus standard video endoscopy for detection of early Barrett's neoplasia: A multicenter, randomized, crossover study in general practice. *Gastrointestinal Endoscopy* 73: 195–203.
- Dawsey SM, Fleischer DE, Wang GQ, et al. (1998) Mucosal iodine staining improves endoscopic visualization of squamous dysplasia and squamous cell carcinoma of the esophagus in Linxian, China. *Cancer* 83: 220–231.
- Dinesen L, Chua TJ, and Kaffes AJ (2012) Meta-analysis of narrow-band imaging versus conventional colonoscopy for adenoma detection. *Gastrointestinal Endoscopy* 75: 604–611.
- Duan XF, Tang P, and Yu ZT (2014) Neoadjuvant chemoradiotherapy for resectable esophageal cancer: An in-depth study of randomized controlled trials and literature review. *Cancer Biological Medicine* 11: 191–201.
- East JE, Vleugels JL, Roelandt P, et al. (2016) Advanced endoscopic imaging: European Society of Gastrointestinal Endoscopy (ESGE) technology review. *Endoscopy* 48: 1029–1045.
- Evans JA, Bouma BE, Bressner J, et al. (2007) Identifying intestinal metaplasia at the squamocolumnar junction by using optical coherence tomography. *Gastrointestinal Endoscopy* 65: 50–56.
- Ezoe Y, Muto M, Uedo N, et al. (2011) Magnifying narrowband imaging is more accurate than conventional white-light imaging in diagnosis of gastric mucosal cancer. *Gastroenterology* 141: 2017–2025.
- Falk GW (2009) Autofluorescence endoscopy. *Gastrointestinal Endoscopy Clinics of North America* 19: 209–220.
- Fennerty MB (1999) Tissue staining (chromoscopy) of the gastrointestinal tract. *Canadian Journal of Gastroenterology* 13: 423–429.
- Freitag CP, Barros SG, Kruel CD, et al. (1999) Esophageal dysplasias are detected by endoscopy with Lugol in patients at risk for squamous cell carcinoma in southern Brazil. *Diseases of the Esophagus* 12: 191–195.

- Fujimoto JG, Pitris C, Boppart SA, and Brezinski ME (2000) Optical coherence tomography: An emerging technology for biomedical imaging and optical biopsy. *Neoplasia* 2: 9–25.
- Garcia-Allende PB, Glatz J, Koch M, et al. (2014) Towards clinically translatable NIR fluorescence molecular guidance for colonoscopy. *Biomedical Optics Express* 5: 78.
- Goetz M, Hoetker MS, Diken M, Galle PR, and Kiesslich R (2013) In vivo molecular imaging with cetuximab, an anti-EGFR antibody, for prediction of response in xenograft models of human colorectal cancer. *Endoscopy* 45: 469–477.
- Gono K (2015) Narrow band imaging: Technology basis and research and development history. *Clinical Endoscopy* 48: 476–480.
- Gora MJ, Sauk JS, Carruth RW, et al. (2013) Tethered capsule endomicroscopy enables less invasive imaging of gastrointestinal tract microstructure. *Nature Medicine* 19: 238–240.
- Gora MJ, Simmons LH, Quénéhervé L, et al. (2016) Tethered capsule endomicroscopy: From bench to bedside at a primary care practice. *Journal of Biomedical Optics* 21: 104001.
- Gora MJ, Quénéhervé L, Carruth RW, et al. (2018) Tethered capsule endomicroscopy for microscopic imaging of the esophagus, stomach, and duodenum without sedation in humans (with video). *Gastrointestinal Endoscopy* 88: 830–840.
- Guo CG, Ji R, and Li YQ (2015) Accuracy of i-scan for optical diagnosis of colonic polyps: A meta-analysis. *PLoS One* 10: e0126237.
- Hanauer SB, Sandborn WJ, Rutgeerts P, et al. (2006) Human anti-tumor necrosis factor monoclonal antibody (adalimumab) in Crohn's disease: The CLASSIC-I trial. *Gastroenterology* 130: 323–333.
- Hartmans E, Tjalma JJJ, Linssen MD, et al. (2018) Potential red-flag identification of colorectal adenomas with wide-field fluorescence molecular endoscopy. *Theranostics* 8: 1458–1467.
- Hatta W, Uno K, Koike T, et al. (2012) A prospective comparative study of optical coherence tomography and EUS for tumor staging of superficial esophageal squamous cell carcinoma. *Gastrointestinal Endoscopy* 76: 548–555.
- Heresbach D, Barrioz T, Lapalus MG, et al. (2008) Miss rate for colorectal neoplastic polyps: A prospective multicenter study of back-to-back video colonoscopies. *Endoscopy* 40: 284–290.
- Inoue H, Sasajima K, Kaga M, et al. (2006) Endoscopic in vivo evaluation of tissue atypia in the esophagus using a newly designed integrated endocytoscope: A pilot trial. *Endoscopy* 38: 891–895.
- Isenberg G, Sivak MV, Chak A, et al. (2005) Accuracy of endoscopic optical coherence tomography in the detection of dysplasia in Barrett's esophagus: A prospective, double-blinded study. *Gastrointestinal Endoscopy* 62: 825–831.
- Joshi BP, Duan X, Kwon R, et al. (2016) Multimodal endoscope can quantify wide-field fluorescence detection of Barrett's neoplasia. *Endoscopy* 48: 1–13.
- Joshi BP, Dai Z, Gao Z, et al. (2017) Detection of sessile serrated adenomas in the proximal colon using wide-field fluorescence endoscopy. *Gastroenterology* 152: 1002–1013.
- Kaise M, Ohkura Y, Iizuka T, et al. (2014) Endocytoscopy is a promising modality with high diagnostic accuracy for gastric cancer. *Endoscopy* 47: 19–25.
- Kaminski MF, Regula J, Kraszewska E, et al. (2010) Quality indicators for colonoscopy and the risk of interval cancer. *The New England Journal of Medicine* 362: 1795–1803.
- Keller R, Winde G, Terpe HJ, Foerster EC, and Domschke W (2002) Fluorescence endoscopy using a fluorescein-labeled monoclonal antibody against carcinoembryonic antigen in patients with colorectal carcinoma and adenoma. *Endoscopy* 34: 801–807.
- Kiesslich R and Neurath MF (2004) Surveillance colonoscopy in ulcerative colitis: Magnifying chromoendoscopy in the spotlight. *Gut* 53: 165–167.
- Kodashima S and Fujishiro M (2010) Novel image-enhanced endoscopy with i-scan technology. *World Journal of Gastroenterology* 16: 1043–1049.
- Kudo SE, Wakamura K, Ikehara N, et al. (2011) Diagnosis of colorectal lesions with a novel endocytoscopic classification—A pilot study. *Endoscopy* 43: 869–875.
- Kuiper T, van den Broek FJC, Naber AH, et al. (2011) Endoscopic trimodal imaging detects colonic neoplasia as well as standard video endoscopy. *Gastroenterology* 140: 1887–1894.

- Kumagai Y, Monma K, and Kawada K (2004) Magnifying chromoendoscopy of the esophagus: In-vivo pathological diagnosis using an endocytoscopy system. *Endoscopy* 36: 590–594.
- Kumagai Y, Kawada K, Yamazaki S, et al. (2009) Endocytoscopic observation of esophageal squamous cell carcinoma. *Digestive Endoscopy* 22: 10–16.
- Li YX, Shen L, Yu HG, Luo HS, and Yu JP (2014) Fujinon intelligent color enhancement for the diagnosis of early esophageal squamous cell carcinoma and precancerous lesion. *The Turkish Journal of Gastroenterology* 25: 365–369.
- Lipman G, Bisschops R, Sehgal V, et al. (2017) Systematic assessment with I-SCAN magnification endoscopy and acetic acid improves dysplasia detection in patients with Barrett's esophagus. *Endoscopy* 49: 1219–1228.
- Maas M, Nelemans PJ, Valentini V, et al. (2010) Long-term outcome in patients with a pathological complete response after chemoradiation for rectal cancer: A pooled analysis of individual patient data. *Lancet Oncology* 11: 835–844.
- Marion JF, Waye JD, Present DH, et al. (2008) Chromoendoscopy-targeted biopsies are superior to standard colonoscopic surveillance for detecting dysplasia in inflammatory bowel disease patients: A prospective endoscopic trial. *The American Journal of Gastroenterology* 103: 2342–2349.
- Martens MH, Maas M, Heijnen LA, et al. (2016) Long-term outcome of an organ preservation program after neoadjuvant treatment for rectal cancer. *Journal of the National Cancer Institute* 108: 171.
- Matsumoto T, Nakamura S, Jo Y, Yao T, and Iida M (2003) Chromoscopy might improve diagnostic accuracy in cancer surveillance for ulcerative colitis. *The American Journal of Gastroenterology* 98: 1827–1833.
- McGill SK, Evangelou E, Ioannidis JPA, Soetikno RM, and Kaltenbach T (2013) Narrow band imaging to differentiate neoplastic and non-neoplastic colorectal polyps in real time: A meta-analysis of diagnostic operating characteristics. *Gut* 62: 1704–1713.
- Mooiweer E, van der Meulen-de Jong AE, Ponsioen CY, et al. (2015) Chromoendoscopy for surveillance in inflammatory bowel disease does not increase neoplasia detection compared with conventional colonoscopy with random biopsies: Results from a large retrospective study. *The American Journal of Gastroenterology* 110: 1014–1021.
- Mori Y, Kudo S, Ikehara N, et al. (2013) Comprehensive diagnostic ability of endocytoscopy compared with biopsy for colorectal neoplasms: A prospective randomized noninferiority trial. *Endoscopy* 45: 98–105.
- Mori Y, Kudo S, Wakamura K, et al. (2015) Novel computer-aided diagnostic system for colorectal lesions by using endocytoscopy (with videos). *Gastrointestinal Endoscopy* 81: 621–629.
- Mori Y, Kudo SE, Chiu P, et al. (2016) Impact of an automated system for endocytoscopic diagnosis of small colorectal lesions: An international web-based study. *Endoscopy* 48: 1110–1118.
- Mouri R, Yoshida S, Tanaka S, et al. (2009) Evaluation and validation of computed virtual chromoendoscopy in early gastric cancer. *Gastrointestinal Endoscopy* 69: 1052–1058.
- Moussata D, Allez M, Cazals-Hatem D, et al. (2018) Are random biopsies still useful for the detection of neoplasia in patients with IBD undergoing surveillance colonoscopy with chromoendoscopy? *Gut* 67: 616–624.
- Nagengast WB, Hartmans E, Garcia-Allende PB, et al. (2019) Near-infrared fluorescence molecular endoscopy detects dysplastic oesophageal lesions using topical and systemic tracer of vascular endothelial growth factor A. *Gut* 68(1): 7–10.
- Nagorni A, Bjelakovic G, and Petrovic B (2012) Narrow band imaging versus conventional white light colonoscopy for the detection of colorectal polyps. *The Cochrane Database of Systematic Reviews* 1: CD008361.
- Nakanishi H, Doyama H, Ishikawa H, et al. (2017) Evaluation of an e-learning system for diagnosis of gastric lesions using magnifying narrow-band imaging: A multicenter randomized controlled study. *Endoscopy* 49: 957–967.
- Nonaka K, Namoto M, Kitada H, et al. (2012) Usefulness of the DL in ME with NBI for determining the expanded area of early-stage differentiated gastric carcinoma. *World Journal of Gastrointestinal Endoscopy* 4: 362–367.
- Park IJ, You YN, Agarwal A, et al. (2012) Neoadjuvant treatment response as an early response indicator for patients with rectal cancer. *Journal of Clinical Oncology* 30: 1770–1776.

- Pfau PR, Sivak MV, and Chak A (2019) Criteria for the diagnosis of dysplasia by endoscopic optical coherence tomography. *Gastrointestinal Endoscopy* 58: 196–202.
- Pierce MC, Javier DJ, and Richards-Kortum R (2008) Optical contrast agents and imaging systems for detection and diagnosis of cancer. *International Journal of Cancer* 123: 1979–1990.
- Pohl J, Lotterer E, Balzer C, et al. (2009) Computed virtual chromoendoscopy versus standard colonoscopy with targeted indigocarmine chromoscopy: A randomised multicentre trial. *Gut* 58: 73–78.
- Pohl J, Schneider A, Vogell H, et al. (2011) Pancolonic chromoendoscopy with indigo carmine versus standard colonoscopy for detection of neoplastic lesions: A randomised two-centre trial. *Gut* 60: 485–490.
- Poneros JM, Brand S, Bouma BE, et al. (2001) Diagnosis of specialized intestinal metaplasia by optical coherence tomography. *Gastroenterology* 120: 7–12.
- Rex DK, Clodfelter R, Rahmani F, et al. (2016) Narrow-band imaging versus white light for the detection of proximal colon serrated lesions: A randomized, controlled trial. *Gastrointestinal Endoscopy* 83: 166–171.
- Rey JW, Kiesslich R, and Hoffman A (2014) New aspects of modern endoscopy. *World Journal of Gastrointestinal Endoscopy* 6: 334–344.
- Rutter MD, Saunders BP, Schofield G, et al. (2004) Pancolonic indigo carmine dye spraying for the detection of dysplasia in ulcerative colitis. *Gut* 53: 256–260.
- Shan J, Liu L, Sun X, et al. (2017) High-definition i-scan colonoscopy is superior in the detection of diminutive polyps compared with high-definition white light colonoscopy. *European Journal of Gastroenterology & Hepatology* 29: 1309–1313.
- Sharma P, Hawes RH, Bansal A, et al. (2013) Standard endoscopy with random biopsies versus narrow band imaging targeted biopsies in Barrett's oesophagus: A prospective, international, randomised controlled trial. *Gut* 62: 15–21.
- Shen B, Zuccaro G Jr., and Gramlich TL (2004) In vivo colonoscopic optical coherence tomography for transmural inflammation in inflammatory bowel disease. *Clinical Gastroenterology and Hepatology* 2: 1080–1087.
- Siegel RL, Miller KD, and Jemal A (2017) Cancer statistics. *A Cancer Journal for Clinicians* 67: 7–30.
- Singh M, Bansal A, Curvers WL, et al. (2011) Observer agreement in the assessment of narrowband imaging system surface patterns in Barrett's esophagus: A multicenter study. *Endoscopy* 43: 745–751.
- Song LMWK, Banerjee S, Desilets D, et al. (2011) Autofluorescence imaging. *Gastrointestinal Endoscopy* 73: 647–650.
- Song J, Zhang J, Wang J, et al. (2015) Meta-analysis of the effects of endoscopy with narrow band imaging in detecting dysplasia in Barrett's esophagus. *Diseases of the Esophagus* 28: 560–566.
- Stoffel EM, Turgeon DK, Stockwell DH, et al. (2008) Missed adenomas during colonoscopic surveillance in individuals with lynch syndrome (hereditary nonpolyposis colorectal cancer). *Cancer Prevention Research* 1: 470–475.
- Sturm MB and Wang TD (2015) Emerging optical methods for surveillance of Barrett's oesophagus. *Gut* 64: 1816–1823.
- Sturm MB, Joshi BP, Lu S, et al. (2013) Targeted imaging of esophageal neoplasia with a fluorescently labeled peptide: First-in-human results. *Science Translational Medicine* 5: 1–9.
- Suter MJ, Gora MJ, Lauwers GY, et al. (2014) Esophageal-guided biopsy with volumetric laser endomicroscopy and laser cautery marking: A pilot clinical study. *Gastrointestinal Endoscopy* 79: 886–896.
- Thompson AJ, Hughes M, Anastasova S, et al. (2017) Position paper: The potential role of optical biopsy in the study and diagnosis of environmental enteric dysfunction. *Gastroenterology & Hepatology* 14: 727–738.
- van den Broek FJC, Fockens P, van Eeden S, et al. (2008) Endoscopic tri-modal imaging for surveillance in ulcerative colitis: Randomised comparison of high-resolution endoscopy and autofluorescence imaging for neoplasia detection; and evaluation of narrow-band imaging for classification of lesions. *Gut* 57: 1083–1089.
- van Rijn JC, Reitsma JB, Stoker J, et al. (2006) Polyp miss rate determined by tandem colonoscopy: A systematic review. *The American Journal of Gastroenterology* 101: 343–350.

- Verna C, Feyles E, Lorenzi L, et al. (2014) I-SCAN targeted versus random biopsies in Barrett's oesophagus. *Digestive and Liver Disease* 46: 131–134.
- Visrodia K, Singh S, Krishnamoorthi R, et al. (2019) Magnitude of missed esophageal adenocarcinoma after Barrett's Esophagus diagnosis: A systematic review and meta-analysis. *Gastroenterology* 150: 599–607.
- Weele ter EJ, van Scheltinga AGTT, Linssen MD, et al. (2016) Development, preclinical safety, formulation, and stability of clinical grade bevacizumab-800CW, a new near infrared fluorescent imaging agent for first in human use. *European Journal of Pharmaceutics and Biopharmaceutics* 104: 226–234.
- Weissleder R and Ntziachristos V (2002) Shedding light onto live molecular targets. *Nature Medicine* 9: 123–128.
- Wolfsen CW, Sharma P, Wallace MB, et al. (2015) Safety and feasibility of volumetric laser endomicroscopy in patients with Barrett's esophagus. *Gastrointestinal Endoscopy* 82: 631–640.
- Yao K, Nagahama T, Matsui T, and Iwashita A (2013) Detection and characterization of early gastric cancer for curative endoscopic submucosal dissection. *Digestive Endoscopy* 25: 44–54.
- Yao K, Uedo N, Muto M, et al. (2016) Development of an E-learning system for the endoscopic diagnosis of early gastric cancer: An international multicenter randomized controlled trial. *eBioMedicine* 9: 140–147.
- Yoshida Y, Goda K, Tajiri H, et al. (2009) Assessment of novel endoscopic techniques for visualizing superficial esophageal squamous cell carcinoma: Autofluorescence and narrow-band imaging. *Diseases of the Esophagus* 22: 439–446.
- Yung DE, Boal Carvalho P, Giannakou A, et al. (2017) Clinical validity of flexible spectral imaging color enhancement (FICE) in small-bowel capsule endoscopy: A systematic review and meta-analysis. *Endoscopy* 49: 258–269.
- Zhang RR, Schroeder AB, Grudzinski JJ, et al. (2017) Beyond the margins: Real-time detection of cancer using targeted fluorophores. *Nature Medicine Reviews Clinical Oncology* 14: 347–364.
- Zhou C, Tsai TH, Lee HC, et al. (2012) Characterization of buried glands before and after radiofrequency ablation by using 3-dimensional optical coherence tomography (with videos). *Gastrointestinal Endoscopy* 76: 32–40.

The optimal imaging window for dysplastic colorectal polyp detection using c-Met targeted fluorescence molecular endoscopy

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ABSTRACT

Rationale

Fluorescence molecular endoscopy (FME) is an emerging technique that has the potential to improve the 22% colorectal polyp detection miss-rate. We determined the optimal dose-to-imaging interval and safety of FME using EMI-137, a c-Met targeted fluorescent peptide, in a population at high-risk for colorectal cancer.

Methods

We performed *in vivo* FME and quantification of fluorescence by multi-diameter single-fiber reflectance, single-fiber fluorescence spectroscopy in 15 patients with a dysplastic colorectal adenoma. EMI-137 was intravenously administered (0.13mg/kg) at a one-, two- or three-hour dose-to-imaging interval (N=3 patients per cohort). Two cohorts were expanded to six patients based on target-to-background ratios (TBR). Fluorescence was correlated to histopathology and c-Met expression. EMI-137 binding specificity was assessed by fluorescence microscopy and *in vitro* experiments.

Results

FME using EMI-137 appeared to be safe and well tolerated. All dose-to-imaging intervals showed significantly increased fluorescence in the colorectal lesions compared to surrounding tissue, with a TBR of 1.53, 1.66 and 1.74 respectively (mean intrinsic fluorescence ($Q \cdot \mu_{a,x}^i$) = 0.035 vs. 0.023mm⁻¹, P<0.0003; 0.034 vs. 0.021mm⁻¹, P<0.0001; 0.033 vs. 0.019mm⁻¹, P<0.0001). Fluorescence correlated to histopathology on a macroscopic and microscopic level, with significant c-Met overexpression in dysplastic mucosa. *In vitro*, a dose-dependent specific binding was confirmed.

Conclusion

FME using EMI-137 appeared to be safe and feasible within a one-to-three hour dose-to-imaging interval. No clinically significant differences were observed between the cohorts, although a one-hour dose-to-imaging interval was preferred from a clinical perspective. Future studies will investigate EMI-137 for improved colorectal polyp detection during screening colonoscopies.

INTRODUCTION

The majority of colorectal cancers (CRC) develop through the adenoma-to-carcinoma sequence.¹ Therefore, early detection of premalignant polyps such as adenomas and sessile serrated polyps could improve patient outcome.² To date, the gold standard for premalignant lesion detection is high definition white-light endoscopy (HD-WLE). Although HD-WLE has significantly contributed to the success of screening for and prevention of CRC, it also has limitations.^{3,4} Factors such as inadequate bowel preparation and skill and expertise of the endoscopist may cause reduced sensitivity, leading to an adenoma detection miss-rate of up to 22% in the general population and 55% in patients with Lynch syndrome.^{5,6} Location (i.e. ascending colon) and morphological characteristics of the adenoma such as small size (i.e. less than 10mm) or flat shape are notorious for higher miss-rates.⁷

This emphasizes the need for a novel imaging technology to reduce the high detection miss rates. Fluorescence molecular endoscopy (FME) makes use of administration of targeted fluorescent tracers that enable the visualization of specific markers that are overexpressed on the target of interest. Combining HD-WLE to visualize morphological mucosal abnormalities with FME to visualize real-time biological characteristics of cells has the potential to improve polyp detection rates.⁸⁻¹⁰

One of the markers that becomes significantly overexpressed as the degree of dysplasia progresses in the colorectal adenoma-to-carcinoma sequence, is c-Met.¹¹ c-Met is a receptor tyrosine kinase that binds to its ligand hepatocyte growth factor and activates several downstream signaling pathways involved in proliferation, motility, migration and invasion.^{11,12} The fluorescently labelled peptide EMI-137 (previously: GE-137), is a water-soluble 26-amino acid cyclic peptide that specifically binds to human c-Met with high affinity.⁸ It has a peak excitation and emission wavelength of 653 and 675nm respectively and favorable pharmacokinetic properties that enable rapid tissue biodistribution, with a background clearance half-life of approximately 2 hours and 30 minutes.

Previously, intravenous (i.v.) administration of EMI-137 three hours prior to colonoscopy showed the potential to detect additional polyps that were initially missed by conventional fiber-based WL colonoscopy.⁸ To expand the clinical applicability of EMI-137 for future phase II/III trials, we determined its optimal dose-to-imaging interval for colorectal polyp detection using FME in parallel to HD-WLE, with quantification of fluorescence using multi-diameter single-fiber reflectance, single-fiber fluorescence (MDSFR/SFF) spectroscopy. In addition, the safety and tolerability of EMI-137 were investigated in a group of patients at high suspicion of CRC.

MATERIALS AND METHODS

Study design

This study was approved by the Ethics Committee 'Foundation Beoordeling Ethiek Biomedisch Onderzoek', The Netherlands. All patients signed written informed consent. Patients were found eligible for study participation if they were 18 years of age or older and scheduled to undergo a diagnostic or therapeutic endoscopy for a colorectal

adenoma containing at least low-grade dysplasia (LGD). Female patients had to be either surgically sterile, post-menopausal or pre-menopausal with a negative urine pregnancy test. The study was registered in the European Clinical Trials Register (2016-002827-27) and conducted according to the Declaration of Helsinki at the University Medical Center Groningen, Groningen, The Netherlands, between October 2017 and September 2018.

***In vivo* endoscopy procedures**

EMI-137 was administered as a single i.v. bolus injection of 0.13mg/kg in a solution of 4.8mg/ml at either one-, two- or three-hours prior to endoscopy, initially with three patients per cohort (Figure 1). All endoscopic procedures were performed by a board-certified gastroenterologist using a clinical HD video-endoscope (CF-HQ190L/I, EVIS EXERA III, Olympus Corporation, Tokyo, Japan) after standard bowel preparation. Therapy consisted of a piecemeal polypectomy, endoscopic mucosal resection (EMR), endoscopic submucosal dissection (ESD) or endoscopic full-thickness resection (eFTR), depending on lesion location, size and characteristics. A 650nm short-pass filter (Chroma Technology Corp., Bellows Falls, VT, USA) was installed in the Olympus xenon light-source (CLV-190, EVIS EXERA III, Olympus Corporation, Tokyo, Japan) to prevent unintended excitation of EMI-137.

Figure 1. Study workflow. (A) EMI-137 was administered intravenously (0.13 mg/kg). (B) After one, two or three hours, real-time fluorescence molecular endoscopy (fiber-based fluorescence camera) and in vivo quantification of fluorescence (multi-diameter single-fiber reflectance, single-fiber fluorescence (MDSFR/SFF) spectroscopy) were performed. Per cohort, three patients were included, with expansion to six patients for the one and two hour cohort based on the interim analysis results.

Fluorescence was visualized *in vivo* using the SurgVision Explorer Endoscope (SVEE, SurgVision B.V., Groningen, The Netherlands), a real-time fluorescence imaging system connected to a flexible fiberscope that can be inserted through the clinical HD video-endoscope. The SVEE consists of a white-light emitting diode and two class III-b lasers optimized for EMI-137 visualization (excitation wavelength: 653nm), which simultaneously produces a white-light, fluorescence and overlay image.

During FME, adenoma visibility was qualitatively described as ‘clearly increased’, ‘mildly increased’ or ‘same as background’ compared to the surrounding normal tissue, by consensus between the gastroenterologist and the investigator. To confirm *in vivo* visualized fluorescence imaging results, fluorescence intensities were subsequently quantified *in vivo* using MDSFR/SFF spectroscopy, an optical technique that corrects for the influence of tissue optical properties and therefore enables determination of the EMI-137 intrinsic fluorescence values (Figure 2A). Briefly, another fiber-bundle was inserted to determine the tissue absorption coefficient and reduced scattering coefficient at the excitation wavelength (650nm) and the emission band of the fluorophore, Cy5** (600–800nm) through direct-contact MDSFR measurements. Subsequently, a SFF spectrum was acquired. After the clinical procedure, the intrinsic fluorescence values ($Q \cdot \mu_{a,x}^f$) of Cy5** in EMI-137 were calculated.^{10,13-15}

Depending on the endoscopy procedure length, multiple FME and MDSFR/SFF spectroscopy measurements were acquired of each lesion and the surrounding normal tissue every 30 minutes. In case *in vivo* MDSFR/SFF measurements could not be acquired due to the lesion location (this fiber-bundle cannot be inverted), measurements were obtained *ex vivo* directly after resection.

Outcome parameters

Safety and tolerability

Vital signs, the injection site and the occurrence of (serious) adverse events based on the common toxicity criteria for adverse events were monitored at regular time-points. Follow-up took place up to 24 to 48 hours after EMI-137 administration.

In vivo target-to-background ratio (TBR) analysis

Regions-of-interest (ROI) of the lesion and surrounding normal colorectal tissue were delineated on one-to-four representative white-light images per patient at approximately the same distance from the fiber, depending on image availability and quality. Subsequently, mean fluorescent intensities (MFI) were calculated as total counts per ROI pixel area using Fiji/ImageJ software (version 2.0.0-rc-68/1.52h). The lesion MFI was divided by the surrounding normal tissue MFI to determine target-to-background ratios (TBR). In addition to the FME image analysis, quantified intrinsic fluorescence values from the *in vivo* MDSFR/SFF spectroscopy measurements were analyzed, correlated to histopathology and similarly a TBR was calculated for each time-point per patient.

Interim analysis

An interim analysis was performed after the first nine patients to evaluate EMI-137 safety data and to determine the TBR using FME and MDSFR/SFF spectroscopy for each cohort. The two cohorts with the optimal TBR were expanded to six patients per cohort. In case of comparable TBRs for all three cohorts, the one- and two-hour cohort were to be expanded to six patients per cohort, as this was preferred from a clinical perspective.

Ex vivo validation

After resection, histopathological processing and examination was performed according to standard clinical protocol of the UMCG by a board-certified gastrointestinal pathologist, blinded for fluorescence imaging results.

Correlation of fluorescence and histopathology

A maximum of four 4µm tissue sections per patient were selected for further analyses, based on section quality and the simultaneous presence of dysplasia and surrounding normal crypts. After xylene deparaffinization, tissue sections were air dried and scanned using the Odyssey CLx fluorescence scanner (LI-COR Biosciences Inc, Lincoln, NE, USA), directly followed by hematoxylin/eosin (H/E) staining, to allow a precise correlation of fluorescence with histology. MFIs were measured as total counts per ROI pixel area for dysplastic mucosa and surrounding normal mucosa based on histological delineation by the pathologist.

Immunohistochemistry

A SP44 rabbit monoclonal primary antibody directed against the membranous and cytoplasmic c-Met epitope was used to perform c-Met immunohistochemistry using the BenchMark ULTRA system (Ventana Medical Systems, Oro valley, Arizona, USA) according to standard clinical protocol of the UMCG. Membrane-localized staining intensities were semi-quantitatively scored as negative (0), weak (1+), moderate (2+) or strong (3+) in dysplastic and surrounding normal colorectal mucosa by the pathologist. A 2+/3+ score was considered positive for c-Met overexpression.

Fluorescence microscopy

Fluorescence microscopy was performed on one representative 4µm tissue section per patient, to evaluate the accumulation of EMI-137 on a microscopic level, as described previously.^{15,16} A DM6000 fluorescence microscope coupled to a DFC360FX camera (Leica Biosystems GmbH, Wetzlar, Germany) was applied using the same settings per magnification on the A (FITC, autofluorescence), I (DAPI, nuclei) and Y5 (Cy5, i.e. EMI-137) filter cube.

Validation side study

Several *in vitro* experiments were performed on a high c-Met overexpressing (HT-29) and a negative c-Met expressing (SW-480) cell line to confirm EMI-137 binding specificity (described in Supplemental Material).¹⁷

Statistical analyses

Descriptive statistics were applied to the patient demographics. For normally distributed data, mean values with standard deviation were used and a Student T test (paired data) was used to test for significance. Not normally distributed data is presented as median values with interquartile range (IQR) and a Wilcoxon (paired data) or Mann-Whitney U (independent data) test was used to test for significance. P-values <0.05 were considered statistically significant. Statistical analyses and graph design were performed using GraphPad Prism (version 8.0, GraphPad Software Inc, San Diego, California, USA).

RESULTS

Study population

A total of 19 patients signed informed consent and were screened for study participation, 16 were found eligible for inclusion. The mean age was 62 years (range 59-73, (Table 1)). All 16 patients received an i.v. bolus injection of 0.13mg/kg EMI-137. Fourteen patients underwent an intended therapeutic procedure (three piecemeal polypectomies, four EMRs, six ESDs and one eFTR), one patient a diagnostic procedure, and in one patient the colonoscopy was prematurely aborted due to the patient's discomfort. Histological assessment of the resected lesions showed that all lesions were tubular adenomas that contained at least LGD. High grade dysplasia (HGD) was present in five tubular adenomas (31.2%) and adenocarcinoma in another three lesions (18.8%; (Table 1)).

Safety and tolerability

No clinically significant changes in vital signs nor any skin abnormalities at the injection site were observed after administration of EMI-137 in any of the patients. One possibly related grade-1 adverse event (hypotension after anesthesia) and one possibly related grade-2 adverse event (mild allergic reaction, multiple hours after EMI-137 administration) were observed. Two serious adverse events occurred, both were iatrogenic perforations of the large intestine, which were considered not related to EMI-137 or any study-related procedures, but to the therapeutic endoscopy procedure (sigmoid perforation during eFTR and rectum perforation during re-ESD in previously performed EMR area).

In vivo endoscopy procedures

FME was performed in 15/16 patients. In one patient the endoscopy procedure was prematurely aborted, as the cecum polyp could not be reached due to the patient's discomfort. This patient received a polypectomy under propofol sedation at a later stage and was replaced in the study. The planned interim analysis after enrolment of the first nine patients showed comparable TBRs for all three time-intervals regarding FME images and MDSFR/SFF spectroscopy measurements (data not shown). The one- and two-hour time interval cohorts were expanded to six patients each, because of clinical preference.

Table 1. Demographics and clinical characteristics

Patient characteristics	N=15	
	No.	%
Sex		
Male	10	66.7%
Female	5	33.3%
Age (years)		
Median (range)	62 (59-73)	
Body-Mass Index (kg/m ²)		
Median (range)	27.7 (21.6-42.7)	
Time cohort		
1 hour	6	40%
2 hours	6	40%
3 hours	3	20%
Endoscopy procedure		
Diagnostic	1	6.7%
Piecemeal polypectomy	3	20%
Endoscopic mucosal resection	4	26.6%
Endoscopic submucosal resection	6	40%
Endoscopic full-thickness resection	1	6.7%
Adenoma size (cm)		
Median (range)	3.0 (1.5-5.5)	
Histology (highest grade)		
Low-grade dysplasia	8	50%
High-grade dysplasia	5	31.2%
Adenocarcinoma	3	18.8%

The dose-to-imaging time intervals of each cohort ranged from 0:54-1:28 hours (one- hour cohort), 1:50-2:33 hours (two-hour cohort) and 2:41-3:20 hours (three-hour cohort). During endoscopy, a total of 16 lesions were detected in 15 patients. The median adenoma size estimated during colonoscopy was 3.0 cm (range 1.5-5.5cm). Fluorescence was qualitatively assessed *in vivo* as clearly increased in 5/16 adenomas (31%), mildly increased in 8/16 adenomas (50%) and the same as the background in 3/16 adenomas (19%) based on FME images. The three lesions that were assessed to have the same fluorescence as the background could be identified using fluorescence due to their morphological characteristics. Representative FME images are shown (Figure 3).

A total of 74 representative FME images acquired at different time-points from the 16 lesions were analyzed to determine the TBR at each time interval. All lesions showed increased fluorescence compared to surrounding normal colorectal tissue, with a median TBR of 2.18 (IQR 0.87), 1.62 (IQR 0.51) and 1.43 (IQR 0.75) for the one-, two- and three-hour dose- to-imaging intervals respectively (Figure 4).

Figure 3. Fluorescence Molecular Endoscopy (FME). Representative FME images of a lesion with surrounding normal tissue for each cohort. Columns from left to right: high-definition (HD) white-light endoscopy image (Olympus CF-HQ 190L/I) and a white-light, fluorescence and overlay image (SurgVision Explorer Endoscope (SVEE)). Histology from top to bottom: adenocarcinoma, adenocarcinoma and low-grade dysplasia.

In addition to the FME TBRs, *in vivo* direct contact MDSFR/SFF spectroscopy measurements were analyzed using a post processing algorithm to quantify the intrinsic fluorescence values by correcting for the optical properties in 14/16 lesions. MDSFR/SFF spectroscopy measurements of the remaining two lesions were performed *ex vivo* directly after adenoma resection, since *in vivo* measurements were not feasible for technical reasons. Quantified intrinsic fluorescence ($Q \cdot \mu_{a,x}^f$) was significantly higher in the adenomas compared to surrounding normal tissue for the one-hour cohort (0.035 ± 0.0023 vs. $0.023 \pm 0.0024 \text{ mm}^{-1}$, $P < 0.0003$), two-hour cohort (0.034 ± 0.0020 vs. $0.021 \pm 0.0014 \text{ mm}^{-1}$, $P < 0.0001$) and three-hour cohort (0.033 ± 0.0023 vs. $0.019 \pm 0.0023 \text{ mm}^{-1}$, $P < 0.0001$; Figure 2B). Quantified fluorescence values in the adenomas remained consistent from one- to at least three-hours. In contrast, a slight decreasing trend was observed for the quantified fluorescence values in normal colorectal tissue (Figure 2B). As a result, the spectroscopy TBRs slightly increased over time, with a mean TBR of 1.53 ± 0.21 , 1.66 ± 0.07 and 1.74 ± 0.16 for the one-, two- and three-hour cohort respectively (Figure 2B, right axis).

When comparing FME TBRs with MDSFR/SFF spectroscopy TBRs, no statistically significant differences were observed, except for the one-hour cohort, in which the FME TBRs were higher than the MDSFR/SFF spectroscopy TBRs (2.18 versus 1.54 , $P = 0.038$; Figure 4). The MDSFR/SFF spectroscopy values that were corrected for the tissue scattering and absorption coefficients showed less variation compared to the values of the FME images.

Figure 4. FME and MDSFR/SFF spectroscopy target-to-background ratios (TBR). Median TBRs of the fluorescence molecular endoscopy (FME) images and MDSFR/SFF spectroscopy data per time-cohort. Histological grade of the adenoma: low-grade dysplasia (LGD; grey), high-grade dysplasia (HGD; blue) and adenocarcinoma (Adenoca; orange). Error bars: median values $\pm$ IQR (FME TBRs) and mean $\pm$ standard deviation (MDSFR/SFF spectroscopy TBRs).

Ex vivo validation

The correlation between fluorescence intensities, histology and c-Met expression was further evaluated *ex vivo*. In accordance with the *in vivo* results, dysplastic or cancerous mucosa showed significantly increased fluorescence compared to the surrounding normal mucosa on 4 μ m tissue sections for each cohort, with a median TBR of 1.69 (IQR 0.49; $P=0.0398$), 1.43 (IQR 0.37; $P=0.0020$) and 1.46 (IQR 0.16; $P=0.0156$) for the one-, two- and three-hour cohort respectively (Figure 5A).

Figure 2. Multidiameter single-fiber reflectance, single-fiber fluorescence (MDSFR/SFF) spectroscopy. (A) Schematic overview of the device. (B) Individual intrinsic fluorescence values of the adenomas and surrounding normal tissue per time-cohort (left y-axis), with mean target-to-background (TBR) ratios (red; right y-axis). Histological grade of the adenoma: low-grade dysplasia (LGD; grey), high-grade dysplasia (HGD; blue) and adenocarcinoma (Adenoca; orange). Error bars: mean values $\pm$ standard deviation.

c-Met immunohistochemical analysis demonstrated a moderate (2+, 72.2%) to strong (3+, 27.8%) membrane overexpression in dysplastic mucosa, whereas normal colorectal mucosa showed a negative (0+, 53.8%) to weak (1+, 46.2%) physiological membrane staining (Figure 5B).

Finally, fluorescence microscopy showed increased fluorescence intensities in the dysplastic or cancerous colorectal crypts compared to surrounding normal colorectal crypts (Figure 5C). The fluorescence signal clearly accumulated in the proximity of the cell membranes of the dysplastic cells. Surrounding normal tissue showed significantly lower fluorescence intensities, with a more stromal localization.

To further investigate EMI-137 binding specificity, *in vitro* experiments were performed. Immunohistochemistry and Western Blotting confirmed c-Met expression in HT-29 cells and minimal c-Met expression in SW-480 cells. Fluorescence microscopy revealed EMI-137 derived fluorescence on the surface of the HT-29 cells, whereas SW-480 cells showed negligible levels of fluorescence (Supplemental Figs. 1A and 1B). Flowcytometry analysis confirmed a dose-dependent membrane binding of EMI-137 in HT-29 cells, in contrast to SW-480 cells. This was supported by a c-Met receptor saturation experiment using EMI-137 and the non-fluorescent unlabeled peptide (AH111972), showing blocking of the c-Met receptors and consequently, significantly lower MFIs only in the HT-29 cells (Supplemental Figure C).

Figure 5. Ex vivo validation of EMI-137 fluorescence. (A) Correlation of mean fluorescence intensities of adenomatous (Dyspl.) and surrounding normal tissue (Norm.) with histology on 4 μ m tissue sections ($N = 35$), with median values $\pm$ IQR (left y-axis) and median target-to-background ratios (right y-axis; red). (B) c-Met membrane expression related to histology, with 0 = negative; 1+ = weak; 2+ = moderate and 3+ = strong membrane expression. (C) Fluorescence microscopy of dysplasia (left) and normal colorectal tissue (right). 40x magnification; DAPI/Hoechst nuclei staining (blue); FITC/autofluorescence (green) and Cy5/EMI-137 derived fluorescence (red).

DISCUSSION

In this study, we demonstrate that FME using 0.13mg/kg EMI-137 administered one-, two- or three-hours prior to colonoscopy appears to be safe and feasible for the detection of colorectal polyps. *In vivo* FME results were confirmed by quantification of intrinsic fluorescence using MDSFR/SFF spectroscopy, showing significantly higher fluorescence in all lesions compared to surrounding normal colorectal tissue at each time interval. No clinically significant differences were observed between the investigated time-cohorts based on extensive in and ex vivo analyses. Therefore, we conclude that FME using EMI-137 can be performed within a one- to three-hour timeframe, which expands the clinical applicability of EMI-137.

The use of FME to improve polyp detection by serving as a ‘red-flag’ endoscopic imaging technique has been previously investigated using different fluorescent tracers, though several factors have hampered further clinical translation.^{9,10} Firstly, the near-infrared fluorescent (NIRF) tracer bevacizumab-800CW targeting vascular endothelial growth factor-A (peak emission 792nm) has unfavorable pharmacokinetics that require a dose-to-imaging interval of 2-3 days, which limits application in a CRC screening population.¹⁰ Secondly, the fluorescent peptide KCC*-FITC (peak emission 519nm), which binds to

sessile serrated adenomas with a V600E point-mutation in BRAF, was evaluated using post-processing software. However, this limits real-time lesion identification in parallel to HD-WLE.⁹ In addition, KCC*-FITC has a peak emission in the visible light spectrum, which reduces specificity due to higher autofluorescence levels compared to NIRF imaging. Besides i.v. administration, topical tracer administration has been evaluated as well during colonoscopy, although this rarely achieves complete mucosal coverage and tracer binding is affected by bowel preparation adequacy.¹⁸ To overcome these limitations, we used a NIRF peptide with a relatively low molecular-weight and therefore favorable pharmacokinetic properties that allows adenoma identification within a timeframe of one- to at least three-hours after i.v. administration.

To identify the most optimal dose-to-imaging interval, quantification of fluorescence is important in early phase clinical FME trials, since fluorescence intensities observed by FME alone are affected by tissue optical properties and technical factors like camera sensitivity, imaging distance and illumination heterogeneity.¹⁹ The addition of MDSFR/SFF spectroscopy as a confirmatory technique provides objective and consistent intrinsic fluorescence values through direct contact measurements that are corrected for the scattering and absorption coefficients of the tissue.^{10,13-15} The significance of this was emphasized in the three adenomas that were qualitatively assessed to have the same fluorescence as the background during FME, while MDSFR/SFF spectroscopy measurements showed a significant difference in intrinsic fluorescence values. Although MDSFR/SFF spectroscopy measurements are currently calculated using a post-processing algorithm, the results of this study further support its development as a complementary technique to FME.

In line with literature, our immunohistochemical analysis confirmed that c-Met is indeed a suitable marker for colorectal adenoma detection, as we observed a clear c-Met membrane overexpression in dysplastic and cancerous mucosa.^{11,12} Although there is heterogeneity in the c-Met overexpression in the adenomas, this did not influence macroscopic fluorescence imaging results (Figure 5). *In vivo* visualized and quantified fluorescence intensities remained consistent over time, indicating specific binding of EMI-137. In addition, the quantified intrinsic fluorescence values did not seem to further increase after one hour (Figure 2B), suggesting that the current dose of 0.13mg/kg already saturates the available c-Met receptors one hour after administration. Interestingly, the background fluorescence levels slightly decreased over time, which was presumably caused by clearance of unbound EMI-137 (clearance half-life: ~2h30m).⁸ A lower tracer dose might further decrease background fluorescence, while still saturating the available c-Met receptors, thereby potentially enhancing TBRs and improving sensitivity.

To date, integrated video-endoscopes that enable highly-sensitive near-infrared fluorescence imaging in parallel to HD-WLE have not been developed. We applied a NIRF endoscopy system that has the potential to be used clinically, since the use of the fiber-based SVEE enabled simultaneous HD-WLE and FME. This required installation of a short-pass filter in the standard Olympus white-light source, which prevents excitation of EMI-137 by the Olympus white-light source due to an overlap with the excitation spectrum of EMI-137. The SVEE fiber-probe consists of 10.000 fibers, allowing sufficient resolution for co-localizing fluorescence intensities to HD-WLE images for the current study design.

However, increasing the number of fibers to 30.000 would improve white-light image quality and facilitate further clinical translation of EMI-137 during screening colonoscopies.^{10,15,16} In addition, the red laser-light was visible on the HD-WLE images from the clinical video-endoscope, influencing HD-WLE image quality. This is a phenomenon that has not been described previously when using bevacizumab-800CW, a fluorescent tracer that emits fluorescence further in the near-infrared light spectrum.^{10,15,16} Alternatively, installation of a short-pass filter in the tip of the endoscope or pulsed acquisition could also prevent this.

In this study, FME was used to visualize fluorescence during endoscopy, while MDSFR/SFF spectroscopy was used to quantify intrinsic fluorescence values by correcting for tissue optical properties. Ultimately, clinicians need a technique that provides real-time objective information, preferably by combining these methods *in vivo*, to reliably guide the endoscopist during screening colonoscopies.

As the aim of this study was to determine the optimal dose-to-imaging interval for EMI-137, only patients with an advanced adenoma detected during a previous colonoscopy were included. As a result, a complete colonoscopy was not performed. In addition, our study population may not be representative for a screening population of patients at average risk of developing CRC. Although our cohorts were relatively small, the 3+3 study design is a commonly used method to acquire information on dosing or timing of a new compound, while limiting the number of exposed patients.²⁰ A future study will need to determine whether indeed EMI-137 can improve the current adenoma detection rate in a larger general screening population.

In conclusion, this study shows that FME using EMI-137 appears to be safe and feasible from a dose-to-imaging interval of one hour to at least three hours. Ultimately, a trade-off may be required between maintaining an adequate TBR for lesion detection and a clinically acceptable dose-to-imaging interval. Our data supports further research on the potential benefit of EMI-137 within this time-frame in phase II/III clinical trials, to investigate the potential improvement in polyp detection rates in a general screening population.

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DISCLOSURE

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REFERENCES

- 1 Arnold M, Sierra MS, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global patterns and trends in colorectal cancer incidence and mortality. *Gut*. 2017;66:683.
- 2 Leslie A, Carey F, Pratt N, Steele R. The colorectal adenoma–carcinoma sequence. *Brit J Surg*. 2002;89:845-860.
- 3 Segnan N, Senore C, Andreoni B, et al. Baseline Findings of the Italian multicenter randomized controlled trial of “once-only sigmoidoscopy” – SCORE. *J Natl Cancer Inst*. 2002;94:1763-1772.
- 4 Atkin W, Wooldrage K, Parkin MD, et al. Long term effects of once-only flexible sigmoidoscopy screening after 17 years of follow-up: the UK flexible sigmoidoscopy screening randomised controlled trial. *Lancet*. 2017;389:1299-1311.
- 5 Stoffel EM, Turgeon KD, Stockwell DH, et al. Missed adenomas during colonoscopic surveillance in individuals with Lynch syndrome (hereditary nonpolyposis colorectal cancer). *Cancer Prev Res*. 2008;1:470-475.
- 6 van Rijn JC, Reitsma JB, Stoker J, Bossuyt PM, van Deventer SJ, Dekker E. Polyp miss rate determined by tandem colonoscopy: a systematic review. *Am J Gastroenterol*. 2006;101:343-350.
- 7 Bressler B, Paszat LF, Vinden C, Li C, He J, Rabeneck L. Colonoscopic miss rates for right-sided colon cancer: A population-based analysis. *Gastroenterology*. 2004;127:452-456.
- 8 Burggraaf J, Kamerling IM, Gordon PB, et al. Detection of colorectal polyps in humans using an intravenously administered fluorescent peptide targeted against c-Met. *Nat Med*. 2015;21:955-961.
- 9 Joshi BP, Dai Z, Gao Z, et al. Detection of sessile serrated adenomas in the proximal colon using wide-field fluorescence endoscopy. *Gastroenterology*. 2017;152:1002-1013.
- 10 Hartmans E, Tjalma J, Linssen MD, et al. Potential Red-Flag Identification of colorectal adenomas with wide-field fluorescence molecular endoscopy. *Theranostics*. 2018;8:1458-1467.
- 11 Bradley CA, Dunne PD, Bingham V, et al. Transcriptional upregulation of c-MET is associated with invasion and tumor budding in colorectal cancer. *Oncotarget*. 2016;7:78932-78945.
- 12 Organ S, Tsao M-S. An overview of the c-MET signaling pathway. *Ther Adv Med Oncol*. 2011;3:S7-S19.
- 13 van Zaane LF, Gamm U, van Driel P, et al. In vivo quantification of the scattering properties of tissue using multi-diameter single fiber reflectance spectroscopy. *Biomed Opt Express*. 2013;4:696-708.
- 14 Hoyer CL, Gamm UA, Sterenborg HJ, Robinson DJ, Amelink A. Method for rapid multidiameter single-fiber reflectance and fluorescence spectroscopy through a fiber bundle. *J Biomed Opt*. 2013;18:107005-107005.
- 15 Tjalma JJ, Koller M, Linssen MD, et al. Quantitative fluorescence endoscopy: an innovative endoscopy approach to evaluate neoadjuvant treatment response in locally advanced rectal cancer. *Gut*. 2020;69:406-410.
- 16 Nagengast WB, Hartmans E, Garcia-Allende PB, et al. Near-infrared fluorescence molecular endoscopy detects dysplastic oesophageal lesions using topical and systemic tracer of vascular endothelial growth factor A. *Gut*. 2019;68:7-10.
- 17 Esfahani SA, Heidari P, Kim SA, et al. Optical imaging of mesenchymal epithelial transition factor (MET) for enhanced detection and characterization of primary and metastatic hepatic tumors. *Theranostics*. 2016;6:2028-38.
- 18 Keller R, Winde G, Terpe H, Foerster E, Domschke W. Fluorescence endoscopy using a fluorescein-labeled monoclonal antibody against carcinoembryonic antigen in patients with colorectal carcinoma and adenoma. *Endoscopy*. 2002;34:801-807.
- 19 Koch M, Symvoulidis P, Ntziachristos V. Tackling standardization in fluorescence molecular imaging. *Nat Photonics*. 2018;12:505-515.
- 20 Le Tourneau C, Lee JJ, Siu LL. Dose escalation methods in phase I cancer clinical trials. *J Natl Cancer Inst*. 2009;101:708-720.

SUPPLEMENTAL MATERIAL

Ex vivo validation side study

To confirm EMI-137 binding specificity, we performed *in vitro* experiments using two human colorectal cell lines: one with high c-Met overexpression (HT-29) and one with negative c-Met expression (SW-480) as a control. Cells were cultured in Gibco RPMI medium with 10% fetal calf serum (Bodinco BV, Alkmaar, The Netherlands). Cell lines were tested to be mycoplasma free by short tandem repeat profiling at Eurofins Genomics (Germany) and were kept in culture for a maximum of 50 passages. Briefly, c-Met membrane expression levels were analyzed in both cell lines by immunohistochemistry using a c-Met specific mouse-monoclonal antibody (sc-514148 clone D-4, Santa Cruz Biotechnology). Fluorescence microscopy was performed as a qualitative visual comparison and to demonstrate EMI-137 binding location at a microscopic level. Fluorescence-activated cell sorting (FACS) analysis was performed to confirm the specificity of the tracer and to measure its binding affinity to the c-Met receptor by a binding and blocking experiment.

C-Met membrane expression levels were analyzed in both cell lines by immunohistochemistry using a c-Met specific mouse-monoclonal antibody (sc-514148 clone D-4, Santa Cruz Biotechnology). For this purpose, cells were incubated in a 1:500 dilution for 1 hour at room temperature. Western blotting was performed to confirm the qualitative c-Met expression on a protein level, as reported previously.¹⁷ The cells were incubated overnight at 4°C with the same antibody (sc-514148) in a 1:500 dilution and a mouse anti-actin monoclonal antibody (Clone: C4, MP biomedical, Santa Ana, CA, USA) in a 1:10.000 dilution as a control. A secondary antibody was used to incubate the membrane (rabbit anti mouse- HRP, DAKO, Santa Clara, CA, USA) in a 1:1.500 dilution for 1 hour.

Subsequently, fluorescence microscopy was performed as a qualitative visual comparison and to demonstrate EMI-137 binding location at a microscopic level. After seven hours incubation in a serum-free phenol-red free RPMI medium at 37°C, cells were washed with 4°C PBS and detached using a Gibco PBS-based enzyme-free cell dissociation buffer at room temperature. Cells were incubated with 10µg of EMI-137 or with medium alone as internal control at 37°C for five minutes. The cells were concentrated after washing steps using a cytospin and stained with 100µl of modified Kaisers glycerin in combination with a 0.5µg/ml Hoechst nuclei staining. Fluorescence microscopy was performed at a 63x magnification with fixed settings using a DM6000 fluorescence microscope coupled to a DFC360FX camera (Leica Microsystems, Wetzlar, Germany).

Fluorescence-activated cell sorting (FACS) analysis was performed to confirm the specificity of the tracer and to measure its binding affinity to the c-Met receptor. Both cell lines were prepared as described previously and subsequently incubated with four different concentrations of EMI-137 (0.5, 5, 50 and 500nM) in room temperature for 20 minutes.¹⁷ In addition, blocking of the c-Met receptor was performed using the non-fluorescent unlabeled peptide (AH111972) in concentrations of 50nM, 500nM, 5µM and 50µM to demonstrate binding affinity. FACS-analyses were performed using the Accuri C6

flow cytometer (BD Biosciences, San Jose, CA, USA). Data analysis was performed using BD Accuri C6 software version 1.0.264.21. All of the above described experiments were repeated in triplicate for both cell lines.

Supplemental Figure 1. *In vitro* experiments. (A) c-Met staining and fluorescence microscopy of the SW-480 (minimal c-Met expression) and HT-29 (high c-Met expression) colorectal cancer cell lines, showing clear c-Met overexpression and specific binding of EMI-137 in the HT-29 cell line. (B) Western Blot analysis confirming high c-Met expression in the HT-29 cell line and minimal-to-negative c-Met expression in the SW-480 cell line. (C) Flowcytometry of both cell lines using increasing concentrations of the unlabeled peptide (AH111972) and EMI-137, showing blocking of the c-Met receptor by the unlabeled peptide (AH111972 + EMI-137, light grey), while EMI-137 only binds to the c-Met expressing HT-29 cell line (dark grey). Error bars represent median values with interquartile range. All experiments were performed in triplicate.

4

C-Met targeted fluorescence molecular endoscopy in Barrett's esophagus patients and identification of outcome parameters for phase-I studies

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ABSTRACT

Rationale

Fluorescence molecular endoscopy (FME) is an emerging technique in the field of gastroenterology that holds potential to improve diagnosis and guide therapy, by serving as a ‘red-flag’ endoscopic imaging technique. Here, we investigated the safety, feasibility and optimal method of administration of EMI-137, targeting c-Met, during FME in Barrett’s esophagus (BE) and report several outcome parameters for early phase FME studies.

Methods

FME was performed in 15 Barrett’s neoplasia patients. EMI-137 was administered to three cohorts of five patients: 0.13 mg/kg intravenously (IV); 0.09 mg/kg IV or topically at a dose of 200 µg/cm BE (n=1) or 100 µg/cm BE (n=4). Fluorescence was visualized *in vivo*, quantified *in vivo* using multi-diameter single-fiber reflectance, single-fiber fluorescence (MDSFR/SFF) spectroscopy and correlated to histopathology and immunohistochemistry. EMI-137 localization was assessed using fluorescence microscopy.

Results

FME using different IV and topical doses of EMI-137 appeared to be safe and correctly identified 16/18 lesions, although modest target-to-background ratios were observed (median range of 1.12–1.50). C-Met overexpression varied between lesions, while physiological expression in the stomach-type epithelium was observed. Microscopically, EMI-137 accumulated around the neoplastic cell membranes. We identified several outcome parameters important for the validation of EMI-137 for FME: 1. the optimal administration route; 2. optimal dose and safety; 3. *in vivo* FME contrast; quantification of intrinsic fluorescence; *ex vivo* correlation of fluorescence, histopathology and target expression; and microscopic tracer distribution.

Conclusions

C-Met targeted FME using EMI-137 may not be the ideal combination to improve BE surveillance endoscopies, however the identified outcome parameters may serve as a valuable guidance for designing and performing future early phase clinical FME studies, independent of which fluorescent tracer is investigated.

INTRODUCTION

Over the past decade, increasing knowledge on molecular and genetic alterations in gastrointestinal diseases along with technical improvements in endoscopy have led to the clinical translation of fluorescence molecular endoscopy (FME).¹ FME is an emerging technique in the field of gastroenterology that enables real-time visualization of disease-specific biomarkers, upregulated proteins or overexpressed receptors during endoscopy.² Therefore, it holds great potential to improve diagnosis and guide therapy, by serving as a 'red-flag' endoscopic imaging technique, which is demonstrated by several landmark papers that have been published on clinical phase I studies investigating the added value of FME for improved (pre)malignant lesion detection and prediction of treatment response.³⁻⁸

There are numerous targeted fluorescent optical imaging agents in the early stages of clinical development. However, translation from bench-to-bedside can be highly challenging, in particular, because of good manufacturing practice production of the imaging agent, safety and toxicity requirements, relatively high initial costs that are involved and insufficient resources or expertise for clinical translation.⁹ In addition, preclinical results that were obtained in mouse tumor xenograft or disease models are very often not sufficiently representative of the human situation in terms of disease pathogenesis, pharmacokinetics, biodistribution and overall safety.

Despite these challenges, several targeted (near-infrared) fluorescent tracers have already been investigated for application in endoscopic imaging for indications such as colorectal polyp⁴⁻⁶ and Barrett's neoplasia⁷ detection or the evaluation of treatment response in inflammatory bowel disease³ or locally advanced rectal cancer patients.⁸ Despite promising results, comparison between studies can be challenging, as different methodologies, analyses and outcome parameters have been used. This is mainly caused by the fact that fluorescence molecular imaging is not yet an established imaging modality and therefore currently no standards and guidelines exist that define FME-study objectives, protocols, outcome parameters or imaging specifications. Such standards and guidelines are especially important for the application of fluorescence molecular imaging in gastroenterology for reasons of obtaining uniformity in executing clinical studies, equipment and evaluation, as it is often challenging to correlate *in vivo* obtained fluorescence imaging results to *ex vivo* histopathology and immunohistochemistry up to a microscopic level.

Therefore, to better validate a fluorescent tracer and to improve comparison between different FME study results, we have performed a c-MET targeting FME study in BE patients following a methodology that is based previous clinical studies in translating molecular fluorescence imaging into the clinic.^{5,7,8,10-13} The aim of such a methodology is to validate a fluorescent tracer in an early phase clinical study by investigating its safety, feasibility and most optimal dose in a well-informed way, without compromising data quality. Here, we demonstrate the practical application and the potential added value of such a methodology, by evaluating the safety, feasibility and optimal method of administration of EMI-137, a fluorescent peptide targeting the c-Met membrane receptor, during FME in patients with Barrett's neoplasia.

METHODS

Study design

This study was a non-randomized, non-blinded, prospective, single-center safety, feasibility and dose-finding study that was conducted at the University Medical Center Groningen (UMCG). Fifteen patients with Barrett's esophagus (BE) aged 18 years or above with a dysplastic lesion (TNM classification: $\leq$ cT1) scheduled to undergo an endoscopic mucosal resection (EMR) were included. Main exclusion criteria were any medical or psychiatric conditions that would compromise a patients' ability to give informed consent, pregnancy, breast feeding and lesions that were not suitable for EMR. All patients gave written informed consent. The study was approved by the medical ethics committee of the UMCG (METc 2016/595; 05-JAN-2017) and registered with ClinicalTrials.gov (Identifier: NCT03205501).

EMI-137, a fluorescent peptide targeting c-Met

C-Met is a transmembrane tyrosine kinase receptor that plays a role in tumor cell migration, invasion, proliferation and angiogenesis^{14,15}, which becomes overexpressed as the degree of dysplasia progresses in BE patients.¹⁶ The fluorescent tracer EMI-137 is a water-soluble 26-amino acid cyclic peptide conjugated to a Cy-5 derived fluorescent dye (peak excitation and emission: 653nm and 675nm) with a high affinity for the human c-Met receptor.⁶

EMI-137 was administered either as an intravenous (IV) bolus injection $\pm$ 2.5 h before the endoscopy procedure (4.8 mg/mL), or topically (100 or 200 μ g/cm BE) during endoscopy using a spraying catheter. Further clarification on the IV dose and topical dose and incubation time is described in the 'Interim analysis' section below. Safety parameters such as heart rate, blood pressure, temperature and the presence of (serious) adverse events were monitored before and at regular intervals after EMI-137 administration.

In vivo study procedures

All EMRs were performed according to standard clinical care based on high-definition, white-light endoscopy (HD-WLE). FME results did not influence clinical decision making. FME was performed using a fiber-bundle consisting of 30,000 coherent fibers (Schöllly Fiberoptic GmbH, Denzlingen, Germany) coupled to the SurgVision Explorer Endoscope (SurgVision BV., Groningen, The Netherlands), a custom-build fluorescence endoscopy platform specifically developed to visualize EMI-137. The fiber-bundle has a field-of-view of 85°, can be inserted through the working channel of the clinical gastroscope and produces a white-light, fluorescence and overlay image for co-localization purposes. To prevent EMI-137 excitation by the Olympus xenon light-source (CLV-190, EVIS EXERA III, Olympus Corporation, Tokyo, Japan), a 650 nm short-pass filter (Chroma Technology Corp., Bellows Falls, VT, USA) was installed.

FME was performed directly after macroscopic identification of a location suspect for neoplasia by HD-WLE (further referred to as 'lesion'). A predetermined set of imaging parameters was used for all patients. In case of topical administration, the esophagus was sprayed with acetylcysteine as an anti-mucolytic (4 mL, 100 mg/mL) and subsequently

rinsed using 0.9% sodium chloride solution. FME was performed before (i.e. as a negative control) and after topical application of EMI-137. Before FME, the esophagus was rinsed after a predetermined incubation time (see 'Interim analysis') using 0.9% sodium chloride solution, to remove any unbound EMI-137. A qualitative assessment was performed on FME images (scaled per patient with minimum-maximum values) to define the fluorescence in the lesion as clearly increased, mildly increased or the same as the surrounding background fluorescence.

Subsequently, direct contact multi-diameter single-fiber reflectance, single-fiber fluorescence (MDSFR/SFF) spectroscopy measurements were acquired *in vivo* in triplicate from the dysplastic lesion and surrounding squamous epithelium and BE if present. MDSFR/SFF spectroscopy enables the quantification of intrinsic fluorescence values by correcting for the tissue optical properties (i.e. scattering and absorption). First, the absorption coefficient and reduced scattering coefficient were measured at the EMI-137 excitation wavelength and over the emission band of the Cy-5 derived fluorescent dye (600-800 nm) through an optical fiber-bundle consisting of two fibers with different diameters. Subsequently, the raw fluorescence spectrum was measured. The intrinsic fluorescence values ($Q \cdot \mu_{a,x}^i$) were then calculated after the endoscopy procedure as described previously.¹⁷⁻¹⁹ All measurements were acquired in triplicate and median values with interquartile range were calculated per location.

In case additional lesions were detected using FME, biopsies were taken after MDSFR/SFF spectroscopy measurements to correlate fluorescence with histology.

Interim analysis

An interim analysis was performed after the inclusion of five patients (0.13 mg/kg EMI-137 IV) to evaluate safety parameters, *in vivo* lesion identification using FME, MDSFR/SFF spectroscopy quantification data and *ex vivo* validation of EMI-137. In case of high background fluorescence limited lesion detection, another five patients were included with a lowered dose (0.09 mg/kg EMI-137 IV), to decrease the amount of unbound circulating EMI-137. In parallel, in case fluorescence in the lesion appeared to be insufficient for adequate lesion detection, the dose could be increased (0.18 mg/kg EMI-137 IV).

If changing the IV dose still resulted in insufficient target-to-background ratios (TBR), the method of administration could be altered to topical application (200 µg/cm BE) to increase the mucosal tracer concentration, aiming to improve TBRs. The dose and incubation time (5 min) could be decreased (100 µg/cm BE) or increased (10 min) respectively, depending on *in vivo* results. In case sufficient TBRs were observed that allowed adequate *in vivo* lesion identification and discrimination from the non-dysplastic surrounding tissue²⁰, a total of a maximum of 20 patients could be included.

Ex vivo validation

To confirm *in vivo* FME results, macroscopic and microscopic fluorescence imaging was performed *ex vivo* during all steps of pathological processing. After resection, a fluorescence image was acquired of each EMR specimen using the PEARL Trilogy Imaging

System (LI-COR Biosciences, Lincoln, Nebraska, USA). A uniquely colored pin was placed at 12 o'clock to ensure identical orientation throughout pathological processing. Subsequently, the EMR-specimens were formalin fixated for 24 h and inked (blue ink on 3 o'clock side, black ink on 9 o'clock side). After histological slicing, the mucosal side of all tissue slices were imaged using the Odyssey CLx flatbed scanner (LI-COR Biosciences, Lincoln, Nebraska, USA). A hematoxylin and eosin (H/E), p53 and caldesmon staining were performed on 4µm tissue sections for routine histopathological analysis according to standard clinical protocol of the UMCG.

c-Met immunohistochemistry was performed using the BenchMark ULTRA system (Ventana Medical Systems, Oro valley, Arizona, USA) using a SP44 rabbit monoclonal primary antibody directed against the membranous and cytoplasmic c-Met (Ventana Medical Systems). C-Met membrane expression was scored negative (0), weak (1+), moderate (2+) or strong (3+) by a board-certified pathologist blinded for the fluorescence imaging results. A score of moderate (2+) or strong (3+) was considered positive for c-Met membrane expression.

Fluorescence microscopy was performed to evaluate the EMI-137 distribution after a Hoechst nuclear staining (0.5 µg/ml Hoechst 33342, Thermo Fisher Scientific, Waltham, Massachusetts, USA), using a DM6000 fluorescence microscope (Leica Biosystems GmbH, Wetzlar, Germany) coupled to a DFC360FX camera. The following filter sets were used: FITC for autofluorescence, DAPI for the nuclei and Cy-5 for EMI-137 derived fluorescence (filter cubes A, I and Y5 respectively) with standard settings for the Cy-5 channel.

In vitro experiments were performed to confirm binding specificity after topical administration of EMI-137 on two esophageal adenocarcinoma cell lines (OE-33, c-Met overexpression and FLO-1, negative c-Met expression). In short, c-MET expression was evaluated by immunohistochemistry and Western Blot analysis. EMI-137 binding localization was evaluated by fluorescence microscopy and cell membrane-binding affinity was confirmed by fluorescence-activated cell sorting analysis in a binding and blocking experiment (see supplementary methods).

Statistical analyses

Normally distributed data was presented as mean values $\pm$ standard deviation (SD). A Student's t-test was used to test for significance for paired or independent data. Non-normally distributed data was presented as median values with interquartile range (IQR). A Mann-Whitney U test (independent data) or Wilcoxon signed rank test (paired data) was used to test for significance. P-values <0.05 were considered statistically significant. GraphPad Prism (version 8.0, GraphPad Software Inc, San Diego, California, USA) was used for data presentation and statistical tests.

RESULTS

Patient characteristics and safety

Fifteen patients with a neoplastic lesion containing at least low-grade dysplasia were included in the study (Table 1). Five patients received 0.13 mg/kg EMI-137 IV, five patients received 0.09 mg/kg EMI-137 IV and five patients received a topical administration of EMI-137 (200 µg/cm BE (N=1) or 100 µg/cm BE (N=4)). IV administration of EMI-137 was performed 02:59±00:17 h (median ± IQR) before endoscopy. There were no (serious) adverse events related to any of the study procedures, nor were there clinically significant changes in vital parameters after the administration of EMI-137.

Table 1. Patient characteristics. A total of 15 Barrett's esophagus patients were included with a lesion containing at least low-grade dysplasia before endoscopic mucosal resection (EMR). A total of 19 lesions were detected using high-definition white-light endoscopy, two of which proved to be benign.

Characteristics	0.13 mg/kg IV (N = 5*) No.	0.09 mg/kg IV (N = 5) No.	Topical (N = 5) No.
Sex			
Male	4	4	5
Female	1	1	0
Age (years)			
Median (range)	57 (54 - 75)	70 (60 – 72)	64 (49 - 76)
Histology Pre-EMR			
LGD	1	1	0
HGD	3	3	2
Adenocarcinoma	1	1	3
Histology EMR-specimen	(N=5 lesions)	(N=6 lesions)	(N=8 lesions)
Benign	0	1	1
LGD	1	0	3
HGD	1	4	1
Adenocarcinoma	3	1	3
Invasion depth			
m2 (lamina propria)	1	1	1
m3 (muscularis mucosae)	4	4	4
pTNM classification (8 th ed.)			
pT1a	3	5	3
pT1b	2	0	2

Intravenous EMI-137 administration

Representative FME images of each dose-cohort are depicted in Figure 1. An overview of the *in vivo* study results of all individual patients is described in Table S1.

In the 0.13 mg/kg IV cohort, 3/4 lesions detected using HD-WLE (N=4 patients) were identified based on increased fluorescence intensities, although a modest contrast was observed with relatively high fluorescence in the surrounding BE and squamous epithelium. One patient was excluded from the *in vivo* analysis as FME could not be performed (Table S1). Quantified intrinsic fluorescence values of the lesions were significantly increased compared to surrounding BE ($P<0.0001$) and squamous epithelium ($P<0.0001$), with a median TBR of 1.50 ± 0.65 and 2.43 ± 0.64 respectively (Figure 2A). All lesions contained dysplasia/EAC on final histopathology. A moderate to strong c-Met membrane expression was observed in the lesions of all four patients that underwent FME (sensitivity 75%). Following our study methodology, we decided to lower the IV dose to 0.09 mg/kg to decrease background signals and improve TBRs.

Figure 1. Representative FME images per dose-cohort. An esophageal adenocarcinoma (EAC), high-grade dysplastic (HGD) and EAC lesion from each cohort are displayed on each row respectively. The high-definition white-light image was acquired with the clinical video-endoscope, whereas the white-light, fluorescence and overlay images were acquired with the fiber. HD = high-definition; IV = intravenous.

Figure 2. Multi-diameter single-fiber reflectance, single-fiber fluorescence (MDSFR/SFF) spectroscopy data. (A) Individual measurements of all intravenously administered patients grouped per cohort. The intrinsic fluorescence ($Q \cdot \mu f_{a,x}$) is defined as the product of the fluorescence quantum yield of the Cy-5 derived fluorescence dye and its absorption coefficient. Both in the 0.13 mg/kg and 0.09 mg/kg cohort, the lesion showed significantly increased fluorescence intensities compared to surrounding BE and squamous epithelium. In vivo MDSFR/SFF spectroscopy could not be performed in two patients of the 0.13 mg/kg cohort due to a malfunction of the device. (B) Individual measurements of all topically administered patients. Before topical application (T_0), there was no statistically significant differences in FME and intrinsic fluorescence values between the lesion, BE and squamous epithelium, while after topical application (T_1), there was a significant difference.

In the 0.09 mg/kg IV cohort, 3/6 lesions detected using HD-WLE (N=5 patients) were identified during FME. Quantified intrinsic fluorescence values of the lesions were significantly increased compared to the surrounding BE ($P<0.0001$) and squamous epithelium ($P<0.0001$), with a median TBR of 1.27 ± 0.15 and 1.71 ± 0.29 respectively (Figure 2A). All three detected lesions had a moderate to strong c-Met membrane expression and proved dysplastic/EAC, whereas the three undetected lesions showed a weak c-Met expression, among which one benign lesion (sensitivity 100% and specificity 100% for the detection of c-Met expression levels using FME). However, lowering the EMI-137 dose did not decrease the background fluorescence compared to the 0.13 mg/kg IV cohort (Figure 2A).

Ex vivo correlation of fluorescence with histology showed a macroscopic colocalization of fluorescence intensities with dysplasia in 19/22 EMR specimens (Figure 3). High fluorescence intensities were observed in benign tissue in 2/22 EMR specimens and in one EMR specimen the lesion could not be discriminated based on fluorescence intensities. Fluorescence microscopy showed that EMI-137 was localized in the proximity of the dysplastic cells (Figure 4A). However, fluorescence imaging of tissue slices and sections showed fluorescence intensities were relatively low in the dysplastic mucosa compared to the submucosa (Figure 3A, red arrows).

Figure 3. *Ex vivo* EMI-137 validation. Representative fluorescence images acquired at every step during pathological processing from an esophageal adenocarcinoma (EAC) endoscopic mucosal resection (EMR) specimen (A) and a mainly benign EMR specimen consisting of BE and squamous epithelium (B) of the same patient. Images are scaled to each other per pathological processing step and can therefore be compared. A fluorescence image, hematoxylin and eosin (HE) staining and c-Met immunohistochemistry staining were performed on subsequent tissue sections for colocalization purposes. Arrowheads indicate relatively low fluorescence in the mucosa (red arrowheads) compared to the submucosa (gray arrowheads). Imaging of EMR specimens: PEARL Trilogy Imaging System; imaging of tissue slices and sections: Odyssey CLx flatbed scanner.

Figure 4. Fluorescence microscopy and c-Met immunohistochemistry. (A) Representative example of different fluorescence microscopy channels of one patient, showing increased fluorescence in the proximity of the adenocarcinoma cells. (B) Representative example of an esophageal adenocarcinoma (EAC) lesion with strong (3+) c-Met expression (B1), a less differentiated EAC lesion with a weak c-Met expression (B2) and foveolar epithelium of the stomach with moderate c-Met expression (B3). The graph shows overall c-Met expression in all 15 patients (B4).

Topical EMI-137 administration

In order to achieve higher mucosal tracer concentrations compared to IV administration, we changed the administration route to topical administration of EMI-137. We first aimed to confirm topical EMI-137 binding specificity *in vitro*. Fluorescence microscopy showed fluorescence on the cell surface of c-Met positive EAC cells after topical administration (OE-33; Figure S1A), with a dose-dependent increase in EMI-137 membrane-binding using flow cytometry, while membrane-binding was blocked by addition of the non-fluorescent unlabeled peptide (AH111972; Figure S1B). In contrast, negligible fluorescence was observed on the c-Met negative EAC cells (FLO-1) using both methods (Figure S1).

Thereafter, EMI-137 was topically administered to another five patients. Autofluorescence levels were negligible compared to fluorescence intensities after topical administration (Figure S2). In the first patient of the topical cohort, both HD-WLE and FME detected one lesion (EAC) with a moderate c-Met expression, showing significantly increased fluorescence in the lesion compared to the surrounding squamous epithelium ($P=0.0286$), with a TBR of 1.64 (200 $\mu\text{g}/\text{cm}$ BE EMI-137, Figure 2B). However, high fluorescence intensities saturated the fluorescence camera, even at the lowest exposure time and gain.

Therefore, we decreased the dose to 100 $\mu\text{g}/\text{cm}$ BE EMI-137 (N=4 patients). Subsequently, six out of seven lesions detected with HD-WLE showed increased fluorescence (Figure 1). Quantified intrinsic fluorescence values were significantly increased for the lesions versus surrounding BE ($P<0.0001$) and squamous tissue ($P<0.0001$), though with a moderate TBR of 1.12 ± 0.11 and 1.33 ± 0.12 respectively (Figure 2B). All lesions contained dysplasia/EAC except for one, which contained benign gastric epithelium, while all showed a moderate-to-high c-Met membrane expression (sensitivity 85.7%). In general, more speckled fluorescence patterns were observed after topical administration, probably as a result of a less homogenous tracer distribution. *Ex vivo* validation results showed that, after topical application, the majority of EMI-137 had been washed away during formalin fixation and paraffin embedding.

Potential clinical added value of c-Met targeted FME

After validation of EMI-137 binding specificity, we assessed the potential clinical benefit of c-Met targeted FME, by evaluating its discriminative potential and the relation with c-Met expression levels. Using FME, 16 out of 18 lesions (89%) in which FME was performed were correctly identified (Figure 5). There were no additional lesions detected. MDSFR/SFF spectroscopy quantification showed increased fluorescence in the lesions compared to surrounding healthy tissue in all cohorts (Figure 2), although a modest contrast was observed with median TBRs ranging from 1.12 to 1.50. In addition, c-Met membrane overexpression was present in 14/17 lesions (82%; preliminary sensitivity 85.7%; specificity 100%) that differentiated, invasive growing lesions (Figure 4B-2). In addition, stomach-type epithelium also showed increased levels of c-Met membrane expression, which complicated lesion detection in the distal part of the esophagus, where the majority of neoplastic BE-lesions are located (Figure 4B-3/4).

Lessons learned: proposal for outcome parameters in phase-I clinical FME studies

Throughout the current study, we identified several outcome parameters that were important to provide reliable data when investigating a novel fluorescent tracer for FME, while limiting the number of patients exposed: 1) the optimal route of administration; 2) the optimal dose and safety; 3) the *in vivo* FME contrast to evaluate potential clinical added value; 4) the *in vivo* quantification of fluorescence with corrections for tissue optical properties; 5) the *ex vivo* validation by correlating fluorescence to histopathology and target expression levels; and 6) the evaluation of the microscopic tracer distribution (Figure 6). All of the above outcome parameters were reported in this phase-I clinical trial investigating FME using EMI-137.

Figure 5. Potential clinical benefit. Of the 18 lesions in which fluorescence molecular endoscopy (FME) was performed, 16 were correctly identified, as 13 dysplastic or early stage esophageal adenocarcinoma (EAC) lesions with c-Met expression were fluorescence-positive and three lesions with a weak c-Met expression were fluorescence-negative (green boxes). One lesion was not identified using FME while expressing c-Met and one fluorescence- and c-Met-positive lesion was identified, despite not containing dysplasia (red boxes). HD-WLE = high-definition white-light endoscopy. *FME could not be performed due to logistical reasons in the 0.13 mg/kg cohort.

Figure 6. Standardized fluorescence molecular endoscopy (FME) methodology. Six crucial outcome parameters were defined that are depicted here (1-6), with corresponding techniques or evaluation methods that should be performed in an early phase clinical study to evaluate a fluorescent tracer. MDRSFR/SFF = multi-diameter single-fiber reflectance, single-fiber fluorescence; EMR = endoscopic mucosal resection; H/E = hematoxylin and eosin.

DISCUSSION

FME is an emerging technique in the field of molecular fluorescence imaging, though standards and guidelines that define FME study designs, objectives and outcome parameters do not exist. We report the first results on FME using EMI-137, a fluorescent peptide targeting the c-Met membrane receptor, in patients with BE, using not only qualitative measurements such as *in vivo* FME or semi-quantification of fluorescence intensities on FME images, but also objective *in vivo* measurements using MDSFR/SFF spectroscopy. Using FME, 16 out of 18 lesions (89%) in which FME was performed were correctly identified related to histology and c-Met expression levels, although no additional lesions were detected. In addition, we have identified several outcome parameters that could guide the validation of a novel fluorescent tracer in phase-I FME studies.

The application of targeted fluorescent tracers has the potential to improve diagnostic and therapeutic endoscopy procedures by potentially guiding biopsies, improving lesion detection rates, restaging and thereby changing clinical decision making. In the emerging field of FME, a structured methodology is of particular importance considering all parameters that influence FME results, such as fiber-based fluorescence imaging, different routes of administration, differences in tissue optical properties, imaging distance and illumination homogeneity, or the fact that piecemeal or part-by-part resections complicate *in vivo* to *ex vivo* correlation. Previously reported standards have been taken into account when identifying the outcome parameters that we propose.^{13,20-22} The clinical implementation of this methodology did not interfere with the clinical endoscopic and pathological workflow.

The optimal route of administration depends on the indication and organ of interest, but also on the pharmacokinetics, half-life and targeting ability of the imaging agent. For upper endoscopy, both IV and topical application are feasible, while topical application is less preferred for lower endoscopy, as it typically involves spraying of a relatively large surface and tracer binding is affected by the success of bowel preparation.^{23,24} A more homogenous tracer distribution can be achieved after IV administration, while spraying enables higher local (mucosal) tracer concentrations, with faster contrast and a substantially lower risk of toxicity (Figure 6).^{7,23,24} For the purpose of validating a fluorescent tracer, IV tracer administration remains a crucial step for *ex vivo* validation and correlation, as topical tracer binding is affected by pathological processing.⁷ Although EMI-137 has favorable pharmacokinetics ($t_{1/2} = 2$ h 30m)⁶, for screening purposes in BE patients topical application would still be preferred from a clinical perspective.

When translating a novel fluorescent tracer into the clinic, the optimal dose should be established, which involves evaluation of the safety and imaging performance. To expose as little patients as possible, a traditional 3+3 dose-escalation design could be incorporated in the study design.²⁵ In general, lower dosages are preferred due to a lower risk of side-effects and toxicity, provided adequate imaging performance is achieved. Here, we slightly deviated from this design, as the safety and feasibility of EMI-137 had previously been demonstrated in a previous study.⁶ However, we incorporated the option in our study design to change the dose based on *in vivo* results and to change the route

of administration in order to achieve higher mucosal tracer concentrations. In addition, the dose and incubation time for topical application as well could be changed, which proved to be highly relevant when the fluorescence camera saturated using a dose of 200 $\mu\text{g}/\text{cm}$ BE EMI-137.

The performance of a fluorescent tracer is influenced by differences in imaging distance, illumination homogeneity and tissue optical properties such as scattering and absorption. MDSFR/SFF spectroscopy is not subject to these factors, as it quantifies intrinsic fluorescence values by correcting for tissue optical properties through direct contact measurements. This provides quantitative values that can assist in evaluating the tracer distribution and support decision making on the optimal dose or dose-to-imaging interval.^{5,8} Here, we showed that MDSFR/SFF spectroscopy provides robust data with small confidence intervals, which assists in an objective comparison of the tracer distribution between dose-cohorts, or even between different FME studies.

This structured FME methodology is designed to fit both the endoscopy and pathology workflow, which allows a macroscopic-to-microscopic validation and correlation of *in* and *ex vivo* results. We specifically demonstrated EMI-137 accumulation in the proximity of the neoplastic cells by comparing EMI-137 derived fluorescence with autofluorescence using fluorescence microscopy, in addition to previous work.⁶ Moreover, fluorescence imaging during each pathological processing step contributed to the decision to change the route of administration, as limited EMI-137 accumulation in the mucosa was observed. Quantification of intrinsic fluorescence values indeed confirmed this resulted in higher mucosal tracer concentrations compared to IV administration of EMI-137 (Figure 2).

The final objective was to evaluate whether c-Met targeted FME using EMI-137 has the potential to improve the neoplastic detection rate in BE-patients, which requires investigating the previously discussed outcome parameters. Ultimately, to validate the clinical efficacy of a promising fluorescent tracer based on the phase-I study results, a subsequent phase-II diagnostic accuracy study would need to define important outcome parameters such as the sensitivity, specificity and negative and positive predictive value of the technique in a large cohort of BE patients. We observed a variable c-Met membrane-expression between the lesions. In addition, the gastric foveolar epithelium also showed a physiological c-Met expression, while the majority of neoplastic lesions is located at the gastroesophageal junction. Secondly, TBRs for discriminating BE-tissue and the neoplasia of 1.12-1.50 may be insufficient for improved lesion detection in a general screening population.

In conclusion, although TBRs could perhaps be improved by further optimization of the tracer dose, we concluded that based on these results, c-Met targeted FME using EMI-137 may not be the ideal marker for application in BE surveillance endoscopies. In addition, we identified six important outcome parameters that fitted the clinical endoscopic and pathological workflow and may serve as a structured guidance for evaluation of a novel fluorescent tracer in early phase FME studies. The addition of quantification of intrinsic fluorescence values through correction for optical properties enables a more

reliable and objective evaluation of the tracer distribution in different patients and between dose-cohorts. The proposed outcome parameters can serve as a valuable guideline for designing and performing future early phase clinical FME studies, independent of which fluorescent tracer is investigated.

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COMPETING INTERESTS

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REFERENCES

- 1 Joshi BP, Wang TD. Gastrointestinal imaging in 2015: Emerging trends in endoscopic imaging. *Nat Rev Gastroenterol Hepatol*. 2016; 13: 72–73.
- 2 Lee JH, Wang TD. Molecular endoscopy for targeted imaging in the digestive tract. *Lancet Gastroenterol Hepatol*. 2016; 1: 147–155.
- 3 Atreya R, Neumann H, Neufert C, Waldner MJ, Billmeier U, Zopf Y, et al.: In vivo imaging using fluorescent antibodies to tumor necrosis factor predicts therapeutic response in Crohn's disease. *Nat Med*. 2014; 20: 313–318.
- 4 Joshi BP, Dai Z, Gao Z, Lee JH, Ghimire N, Chen J, et al. Detection of Sessile Serrated Adenomas in the Proximal Colon Using Wide-Field Fluorescence Endoscopy. *Gastroenterology*. 2017; 152: 1002–1013.
- 5 Hartmans E, Tjalma JJJ, Linssen MD, Allende PBG, Koller M, Jorritsma-Smit A, et al. Potential Red-Flag Identification of Colorectal Adenomas with Wide-Field Fluorescence Molecular Endoscopy. *Theranostics*. 2018; 8: 1458–1467.
- 6 Burggraaf J, Kamerling IMC, Gordon PB, Schrier L, de Kam ML, Kales AJ, et al. Detection of colorectal polyps in humans using an intravenously administered fluorescent peptide targeted against c-Met. *Nat Med*. 2015; 21: 955–961.
- 7 Nagengast WB, Hartmans E, Garcia-Allende PB, Peters FTM, Linssen MD, Koch M, et al. Near-infrared fluorescence molecular endoscopy detects dysplastic oesophageal lesions using topical and systemic tracer of vascular endothelial growth factor A. *Gut*. 2019; 68: 7–10.
- 8 Tjalma JJJ, Koller M, Linssen MD, Hartmans E, de Jongh S, Jorritsma-Smit A, et al. Quantitative fluorescence endoscopy: an innovative endoscopy approach to evaluate neoadjuvant treatment response in locally advanced rectal cancer. *Gut* 2019; epub ahead of print.
- 9 Reilly RM, Lam K, Chan C, Levine M. Advancing Novel Molecular Imaging Agents from Preclinical Studies to First-in-Humans Phase I Clinical Trials in Academia—A Roadmap for Overcoming Perceived Barriers. *Bioconjug Chem*. 2015; 26: 625–632.
- 10 van Dam GM, Themelis G, Crane LMA, Harlaar NJ, Pleijhuis RG, Kelder W, et al. Intraoperative tumor-specific fluorescence imaging in ovarian cancer by folate receptor- α targeting: first in-human results. *Nat Med*. 2011; 17: 1315–1319.
- 11 Harlaar NJ, Koller M, de Jongh SJ, van Leeuwen BL, Hemmer PH, Kruijff S, et al. Molecular fluorescence-guided surgery of peritoneal carcinomatosis of colorectal origin: a single-centre feasibility study. *Lancet Gastroenterol Hepatol*. 2016; 1: 283–290.
- 12 Lamberts LE, Koch M, de Jong JS, Adams ALL, Glatz J, Kranendonk MEG, et al. Tumor-Specific Uptake of Fluorescent Bevacizumab-IRDye800CW Microdosing in Patients with Primary Breast Cancer: A Phase I Feasibility Study. *Clin Cancer Res*. 2017; 23: 2730–2741.
- 13 Koller M, Qiu S-Q, Linssen MD, Jansen L, Kelder W, de Vries J, et al. Implementation and benchmarking of a novel analytical framework to clinically evaluate tumor-specific fluorescent tracers. *Nat Commun*. 2018; 9: 37–39.
- 14 Bradley CA, Salto-Tellez M, Laurent-Puig P, Bardelli A, Rolfo C, Tabernero J, et al. Targeting c-MET in gastrointestinal tumours: rationale, opportunities and challenges. *Nat Rev Clin Oncol*. 2017; 14: 562–576.
- 15 Organ SL, Tsao M-S. An overview of the c-MET signaling pathway. *Therapeutic Advances in Medical Oncology*. 2011; 3: S7–S19.
- 16 Herrera LJ, El-Hefnawy T, Queiroz de Oliveira PE, Raja S, Finkelstein S, Gooding W, et al. The HGF Receptor c-Met Is Overexpressed in Esophageal Adenocarcinoma. *Neoplasia*. 2005; 7: 75–84.
- 17 van Leeuwen-van Zaane F, Gamm UA, Van Driel PBAA, Snoeks TJA, de Bruijn HS, van der Ploeg van den Heuvel A, et al. In vivo quantification of the scattering properties of tissue using multi-diameter single fiber reflectance spectroscopy. *Biomed Opt Express*. 2013; 4: 696–13.
- 18 Hoy CL, Gamm UA, Sterenberg HJCM, Robinson DJ, Amelink A. Method for rapid multidiameter single-fiber reflectance and fluorescence spectroscopy through a fiber bundle. *J Biomed Opt*. 2013; 18: 107005.
- 19 Middelburg TA, Hoy CL, Neumann HAM, Amelink A, Robinson DJ. Correction for tissue optical properties enables quantitative skin fluorescence measurements using multi-diameter single fiber reflectance spectroscopy. *J Dermatol Sci*. 2015; 79: 64–73.

- 20 Tummers WS, Warram JM, van den Berg NS, Miller SE, Swijnenburg R-J, Vahrmeijer AL, et al. Recommendations for reporting on emerging optical imaging agents to promote clinical approval. *Theranostics*. 2018; 8: 5336–5347.
- 21 Koch M, Ntziachristos V. Advancing Surgical Vision with Fluorescence Imaging. *Annu Rev Med*. 2016; 67: 153–164.
- 22 Hoogstins C, Burggraaf JJ, Koller M, Handgraaf H, Boogerd L, van Dam G, et al. Setting Standards for Reporting and Quantification in Fluorescence-Guided Surgery. *Mol Imaging Biol*. 2019; 21: 11–18.
- 23 Pierce MC, Javier DJ, Richards-Kortum R. Optical contrast agents and imaging systems for detection and diagnosis of cancer. *Int J Cancer*. 2008; 123: 1979–1990.
- 24 Goetz M, Hoetker M, Diken M, Galle P, Kiesslich R. In vivo molecular imaging with cetuximab, an anti-EGFR antibody, for prediction of response in xenograft models of human colorectal cancer. *Endoscopy*. 2013; 45: 469–477.
- 25 Le Tourneau C, Lee JJ, Siu LL. Dose escalation methods in phase I cancer clinical trials. *J Natl Cancer Inst*. 2009; 101: 708–720.

SUPPLEMENTAL MATERIAL

Supplemental methods

EMI-137 binding specificity in vitro

In vitro EMI-137 binding experiments were performed to confirm binding specific after topical administration of EMI-137. Two esophageal adenocarcinoma cell lines were used: one with high overexpression of c-Met (OE-33) and one with a negligible c-Met expression (FLO-1). Cell culturing was performed in Gibco RPM I medium with 10% fetal calf serum (Bodinco BV, Alkmaar, The Netherlands). All experiments described in this *in vitro* experiment section were performed in triplicate. C-Met expression levels were confirmed by immunohistochemistry. Cells were incubated with a mouse-monoclonal c-Met primary antibody (sc-514148 clone D-4, 1:500, Santa Cruz Biotechnology) at room temperature for one hour, with a secondary antibody (rabbit-anti-mouse-HRP, 1:100, DAKO, Santa Ana, CA, USA) at room temperature for 30 minutes and a tertiary antibody (goat-anti-rabbit-HRP, 1:100, DAKO). Subsequently, Western Blotting was performed to confirm c-Met expression on a protein level, as previously reported.¹ The same c-Met mouse-monoclonal antibody (sc-514148, 1:500) was used to incubate the blots overnight at 4°C. A mouse anti-actin monoclonal antibody (Clone: C4, 1:10.000, MP biomedical, Santa Ana, CA, USA) was used as a control.

Fluorescence microscopy was performed to evaluate EMI-137 binding specificity after topical application of EMI-137. Cells were incubated in a serum-free phenol-red free RPMI medium for seven hours at 37°C, washed with PBS at 4°C and detached using a Gibco PBS-based enzyme-free cell dissociation buffer at room temperature. A total of 10µg EMI-137 was used to incubate cells, or cells were incubated solely with the medium as a control for five minutes at 37°C. A cytospin was used to concentrate cells after washing steps. Modified Kaisers glycerin combined with a Hoechst nucleus staining (0.5µg/ml) was used for staining. A DM6000 fluorescence microscope coupled to a DFC360FX camera (Leica Microsystems, Wetzlar, Germany) was used on a 63x magnification with fixed settings.

To confirm EMI-137 membrane binding, flowcytometry (i.e. fluorescence-activated cell sorting) analysis was performed. Cells were prepared as described previously.²⁵ Four different concentrations of EMI-137 (0.5, 5, 50 and 500 nM) and four different concentrations of the unlabeled peptide AH111972 (50nM, 500nM, 5µM and 50µM) were used to incubate cells to demonstrate binding affinity (EMI-137 alone) or blocking of the c-Met receptor (EMI-137 and AH111972 combined). Flowcytometry analysis were performed using the Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA) with software version 1.0.264.21.

Supplemental Figures

A Immunohistochemistry and Fluorescence Microscopy

B Flow Cytometry

Figure S1. In vitro confirmation of EMI-137 binding specificity. (A) c-Met immunohistochemistry and fluorescence microscopy of the c-Met negative (FLO-1) and c-Met positive esophageal adenocarcinoma cell lines, showing negligible fluorescence and specific binding respectively. (B) Flow Cytometry experiment on both cell lines showing a dose-dependent specific membrane binding after topical administration of EMI-137, that was blocked by addition of the unlabeled c-Met specific peptide AH111972.

Figure S2. Representative autofluorescence image. White-light and fluorescence fiber image before topical application of EMI-137 (left) demonstrating the autofluorescence of the tissue, versus the same lesion after topical application of EMI-137, 5 minutes incubation time and rinsing using 0.9% sodium chloride solution (right). All fluorescence images are scaled equally and acquired of the same lesion using identical image acquisition parameters and can therefore be compared.

Supplemental Table

	Morphology	Histology	HD-WLE	FME	c-Met
Patient 1	Flat	HGD	Visible	Mildly increased	Moderate
Patient 2	Flat	Adenoca	Visible	Not applicable*	Weak
Patient 3	Flat	LGD	Visible	Same as background	Strong
Patient 4	Protruding	Adenoca	Visible	Increased	Moderate
Patient 5	Elevated	Adenoca	Visible	Increased	Moderate
Patient 6	Elevated	HGD	Visible	Increased	Moderate
Patient 7	Elevated	HGD	Visible	Increased	Moderate
Patient 8	Flat	HGD	Visible	Same as background	Weak
Patient 9 #1	Flat	HGD	Visible	Same as background	Weak
Patient 9 #2	Flat	Benign	Visible	Same as background	Weak
Patient 10	Protruding	Adenoca	Visible	Increased	Strong
Patient 11	Elevated	Adenoca	Visible	Increased	Moderate
Patient 12	Elevated	HGD	Visible	Increased	Moderate
Patient 13 #1	Protruding	Adenoca	Visible	Mildly increased	Strong
Patient 13 #2	Flat	LGD	Visible	Mildly increased	Moderate
Patient 14 #1	Flat	LGD	Visible	Same as background	Moderate
Patient 14 #2	Protruding	LGD	Visible	Mildly increased	Moderate
Patient 15 #1	Elevated	Adenoca	Visible	Increased	Strong
Patient 15 #2	Protruding	Benign	Visible	Mildly increased	Moderate

* FME could not be performed as the gastroscope that should be coupled to the Olympus white-light source with the fluorescence filter installed was unavailable.

Table S1. In vivo study results. HD-WLE = high-definition, white-light endoscopy; FME = fluorescence molecular endoscopy; HGD = high-grade dysplasia; LGD = low-grade dysplasia; Adenoca = adenocarcinoma.

PART II

FLUORESCENCE-GUIDED SURGERY AND PATHOLOGY

5

Molecular fluorescence-guided surgery of peritoneal carcinomatosis of colorectal origin: a narrative review

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ABSTRACT

Patients with peritoneal carcinomatosis (PC) from colorectal origin may undergo cytoreductive surgery (CRS) with hyperthermic intraperitoneal chemotherapy (HIPEC) as a curative approach. One major prognostic factor that affects survival is completeness of cytoreduction. Molecular Fluorescence Guided Surgery (MFGS) is a novel intraoperative imaging technique that may improve tumor identification in the future, potentially preventing over- and undertreatment in these patients. This narrative review outlines a chronological overview of MFGS development in patients with PC of colorectal origin.

INTRODUCTION

Colorectal cancer (CRC) is one of the most common cancers worldwide, with an incidence of 40 patients per 100 000 population and a mortality rate of 15 per 100 000 persons.^{1,2} Of these patients, 8-25% develop peritoneal carcinomatosis (PC).³⁻⁶ Over the past decades, the treatment of PC of colorectal origin has evolved considerably, from palliative care toward a more successful treatment approach with curative intent.^{7,8} In particular, the introduction of cytoreductive surgery (CRS) combined with hyperthermic intraperitoneal chemotherapy (HIPEC) has contributed significantly to this change.^{9,10} After surgical cytoreduction of all macroscopic tumor tissue, the abdominal cavity is perfused with heated chemotherapy in order to eliminate remaining microscopic disease. Up to date, only one randomized clinical trial has been performed studying patients with PC of colorectal origin. A median overall survival of 22 months was seen for patients after undergoing CRS in combination with HIPEC, compared to 13 months for patients receiving only systemic chemotherapy with or without palliative surgery.^{11,12} The authors report a 5-years survival of 43% for patients in whom all macroscopic tumor was removed, compared to 0% for patients in whom residual lesions of more than 2.5 mm were left behind.¹¹ These findings emphasize the importance of patient selection and a macroscopically complete cytoreduction, mainly because incomplete cytoreduction followed by HIPEC does not contribute to a prolonged survival, but potentially does introduce a high risk of postoperative complications, an extensive rehabilitation period and subsequently decreased quality of life.¹²⁻¹⁵

Although the technical quality of the complete CRS/HIPEC procedure has improved, still up to 88% of the patients undergoing CRS and HIPEC for PC of CRC develop recurrent disease within 2 years.¹⁶ Currently, many imaging modalities are available for preoperative staging, such as ultrasonography, computed tomography (CT), magnetic resonance imaging (MRI), or positron emission tomography (PET) scans. Unfortunately, all of these imaging modalities are insufficient for the preoperative assessment of tumor load, or determination of a preoperative Peritoneal Cancer Index (PCI), the most important staging system in PC. CT, MRI, and fluorodeoxyglucose (FDG-) PET scans have a poor sensitivity and specificity to estimate PCI by detection of individual tumor deposits, due to the limited spatial resolution.¹⁷ For example, the detection of individual peritoneal deposits using a CT-scan varies from 9.1 to 24.3% for tumor sizes <1 cm, to up to 59.3-66.7% for tumor size of over 5 cm.¹⁸ These results are in accordance with other previous studies.¹⁹⁻²¹ Current hybrid PET/CT scanners have a limited spatial resolution of 5-8 mm,²² whereas MRI seems to be more promising in detecting peritoneal lesions.²³

For intraoperative differentiation between benign and malignant lesions, surgeons currently depend on visual and tactile inspection only. Unfortunately, the human eye and palpation are not competent enough to detect molecular changes in intra-abdominal lesions that have the same color and physical properties, or to distinguish tumor lesions from benign scar tissue originating from previous surgery. Today, to the best of our knowledge, no intraoperative imaging modalities provided by the more classical modalities like PET, are available to assist in the real-time identification of peritoneal cancer deposits, loco-regional metastases, and tumor-positive resection margins.

Considering the high tumor recurrence rates after the CRS and HIPEC procedure, there is a clear need for an imaging modality that can aid the oncological surgeon in the differentiation between tumor and benign tissue intraoperatively. In recent years, optical molecular imaging using tumor-targeted fluorescence tracers has emerged as a promising imaging technique for real-time guidance in oncological surgery.²⁴⁻²⁶ This technique can be applied intraoperatively to serve as a “red-flag” imaging technique to assist in optimal tumor identification. Improved detection of tumor tissue could not only help attain a more complete cytoreduction, but might also facilitate tailored surgery, avoiding unnecessary resections of benign lesions and organs.

This narrative review explains the principles of intraoperative optical molecular imaging and provides a chronological overview of the development of Molecular Fluorescence Guided Surgery (MFGS) in patients with PC of colorectal origin.

2. PRINCIPLES OF INTRAOPERATIVE OPTICAL MOLECULAR IMAGING

In colorectal surgery, as in surgical oncology in general, radical surgery and tumor-free resection margins are essential for optimizing patient prognosis. Optical molecular imaging using fluorescence imaging agents can provide real-time intraoperative feedback with high resolution, that is in concordance with the natural surgical field of view of a surgeon and based on the molecular characteristics of the tissue (Figure 1). The technique makes use of non-ionizing imaging agents and can be implemented relatively easily in the current surgical workflow.

Over the past decades, there has been an increased interest in the clinical application of optical molecular imaging using fluorescence imaging agents. Fluorescence occurs when a photon or fluorescent dye absorbs light at a certain wavelength, subsequently triggering the release of a photon with a longer wavelength.²⁷ The quality of fluorescence imaging is

Figure 1. Concept of molecular fluorescence guided surgery (MFGS). Prior to surgery a fluorescent target tracer is injected intravenously (A). During the operation the surgeon will receive real-time feedback by a molecular fluorescence camera in the detection tumor tissue (B). Unpublished figure from previously published study Harlaar et al.¹⁰⁷

influenced by different factors such as changes in photon directions (i.e., scattering) and absorption of photons by the tissue. Multiple tissue components play an important role in fluorophore absorption, with the most relevant being hemoglobin, water, and lipids. As the scattering and absorption properties of tissue are lower in light with longer wavelengths, the near-infrared (NIR) light spectrum (700-900 nm) is considered the optimal clinical diagnostic window for fluorescence imaging.²⁸ These characteristics result in deeper penetration depths of up to one to three centimeters that can be obtained in the NIR light spectrum, leading to higher signal-to-background (SBR) ratios compared to the visible light spectrum (i.e., red- green-blue white-light, 380-700 nm).^{29, 30}

NIR fluorescence light is invisible to the human eye and therefore special imaging devices are required to visualize fluorescence during surgery. In general, these camera systems are equipped with two different light sources: a white-light source and a NIR fluorescence light source. Due to the use of a dichroic mirror and specific filters installed in the camera system itself, the visible light derived from the tissue can be separated from NIR fluorescence light, which enables simultaneous imaging of both visible and NIR fluorescence light. Next to that, an overlay of fluorescence signals can be projected on the “normal” white- light images by use of computer software.²⁷ In the operating theatre, all three images can be displayed on monitors at the same time, providing real-time imaging related to the natural surgical field of view (Figure 2). Currently, there are several different intraoperative NIR fluorescence imaging devices available for research and clinical use.³¹⁻³⁸

Figure 2. Intraoperative imaging with white-light, NIR fluorescence and the overlay of both. Intraoperative imaging of a patient with PC of colorectal origin following intravenous administration of 4.5 mg of the fluorescent tracer bevacizumab-800CW targeting VEGF-A. A white-light image (A), NIR fluorescence image (B), and overlay of both (C) clearly show fluorescent signals at the location of a clinically suspect peritoneal lesion. Back-table imaging directly after surgery of a different peritoneal lesion of the same patient is depicted (D-F). Both peritoneal lesions proved to be tumor metastasis upon final histopathology. Unpublished figures from previously published study Harlaar et al.¹⁰⁷

Fluorescence signals in tissue arise by either an endogenous tissue component (i.e., autofluorescence), or an intravenously administered exogenous optical contrast agent. At present, various types of optical contrast agents are available enabling intraoperative imaging, which can roughly be divided into non-targeted and targeted imaging agents.

The effect of non-targeted imaging agents is mainly based on vascularization and perfusion (i.e., also the so-called Enhanced Permeability and Retention (EPR) effect), whereas targeted imaging agents specifically bind to a receptor or protein that is present in a tumor cell. Due to genetic alterations that occur in cancer development, various receptors and proteins become upregulated, which can potentially be used as targets for imaging purposes.³⁹ Prior to developing such targeted imaging agents, it is essential to identify which genes or proteins become upregulated for each specific tumor type.^{40, 41}

3. UPREGULATED GENES AND PROTEINS RELATED TO CRC

The potential application of targeted imaging agents for intraoperative tumor visualization is dependent on the expression levels of biomarkers. A biomarker is a specific component present on or secreted by the tumor cell itself.

Most colorectal cancers are thought to develop via the “adenoma-to-carcinoma sequence”, arising from normal cells through the stepwise asset of different genetic alterations.^{42, 43} In these expressed genes different functional categories can be identified: genes related to proliferation and metabolic rates, to cell adhesion and communication, to transcription and mitosis regulation, or to apoptosis.^{44, 45} Knowing which biomarkers are encoded by which genes is important when searching for which target to develop a fluorescence imaging agent for.

Cardoso et al presented a list of 128 different genes that were found to be upregulated in CRC compared to normal colorectal tissue.⁴⁴ Since protein expression is not always synchronously upregulated, not all of these genes result in overexpression of the related proteins or receptors. Previously, an extensive literature search has been performed on this specific list of genes, in order to identify which genes gave an upregulation of the related proteins or receptors as confirmed by immunohistochemical analysis.⁴⁶ As a result, 29 targets were identified, that could be used for imaging purposes during CRC surgery.

4. TARGET SELECTION CRITERIA (TASC)

To select the most optimal target for imaging purposes from this large set of upregulated biomarkers, the Target Selection Criteria (TASC) scoring system was developed.⁴⁶ The aim of the TASC was to improve the selection of suitable biomarkers for tumor-targeted imaging of all types of cancer. Seven of the most relevant target characteristics were identified based on literature, that each could be scored with 0-6 points. The following characteristics were identified by which a biomarker is validated: i) extracellular biomarker localization—either on the cell membrane or in close proximity of the tumor cell; ii) expression pattern; iii) tumor-to- healthy tissue ratio (T/N); iv) percentage of positive tumors; v) reported successful use of the biomarker in *in vivo* imaging studies; vi) enzymatic activity; and vii) internalization.⁴⁶ Based on extensive testing of the TASC on a variety of biomarkers, cut-off values were

determined for target selection. A total score of 18 or more indicates that a biomarker can be considered a potential candidate for tumor-targeted imaging.

As mentioned before, 29 targets were identified that may be used as potential targets for intraoperative imaging of CRC.⁴⁶ Using the TASC-scoring system, six biomarkers were considered the most promising: Epithelial Cell Adhesion Molecule (EpCAM), CXC Chemokine Receptor 4 (CXCR4), Mucin 1 (Muc1), Matrix MetalloProteinases (MMPs), Epidermal Growth Factor Receptor (EGFR), and Carcino-Embryonic Antigen (CEA). Although the Vascular Endothelial Growth Factor-A (VEGF-A) scored a total of 17 points, it was still considered a suitable potential target as well, given the extensive experience there already is in VEGF-A targeted imaging. For the clinical translation of these seven suitable biomarkers, specific fluorescence imaging agents need to be available to facilitate MFGS of CRC and PC of colorectal origin.

5. FLUORESCENCE IMAGING AGENTS

As stated before, fluorescence imaging agents or probes that can be used for MFGS can roughly be divided into two categories: non-targeted fluorescent probes and targeted fluorescent probes. The main difference between these two categories is based on their mechanism of action (MOA).

5.1 Non targeted fluorescent probes

Non-targeted fluorescent probes accumulate “passively” in solid tumors due to physiological properties such as increased angiogenesis, pressure differences, and high metabolic activity (Figure 3A). It is commonly known that the majority of solid tumor cells stimulate angiogenesis and therefore are highly vascularized. This feature combined with the lack of efficient lymphatic drainage results in more accumulation in tumor tissue compared to normal surrounding tissue, thereby enhancing contrast and enabling a differentiation between the tumor and surrounding tissue. This phenomenon is also known as the EPR effect.

Many non-targeted fluorescent probes have already been used in humans to enhance contrast during surgery in a variety of different indications, such as for example fluorescein for retinal fluorescein angiography, indocyanine green (ICG) for liver perfusion⁴⁷⁻⁴⁹ and lymph node detection,^{50, 51} or methylene blue for sentinel lymph node detection in breast cancer patients.^{52, 53}

ICG is the most commonly known fluorescent probe, which was already approved by the Food and Drug Administration (FDA) back in 1959. ICG has several advantages over to fluorescein as it only fluoresces in the NIR light spectrum (instead of the visible light spectrum) and therefore is less influenced by tissue optical properties such as scattering and absorption. As ICG binds to plasma proteins, it has a negligible toxicity and is excreted rapidly by the liver into the bile, with a plasma half-life of only 3-4 min.^{54, 55} These features make ICG a very attractive contrast agent for assessment of macro- and micro-circulatory status of different organs based on its intravascular distribution.⁵⁶

Figure 3. Fluorescence imaging probes. Overview of fluorescent imaging probes with different mechanisms of action. The effect of non-targeted fluorescent probes is based on tissue distribution by perfusion (A), whereas antibody-based (B), peptide-based (C), and small molecule-based (D) imaging enables targeted fluorescence imaging through binding to specific receptors or proteins overexpressed by the tumor. Smart activatable fluorescent probes are activated upon cleavage by specific enzymes or proteases secreted by the tumor (E), whereas pH activated probes become fluorescent through a change in molecular structure due to the characteristic acidic environment of a tumor (F).

Ever since its first clinical application in hepatology for liver condition monitoring in 1957,⁵⁷ it has been widely applied and studied to visualize perfusion in ophthalmology for identification of retinal blood vessels, in cardiac bypass surgery for evaluation of anastomoses and for monitoring cardiac output.⁵⁸⁻⁶² More recent studies have reported the potential application of ICG for intraoperative fluorescence angiography in a broad range of other indications such as neuro-, coronary-, reconstructive-, liver-, and vascular surgery.^{59, 63-70}

The first potential application of ICG-based fluorescence imaging in patients with peritoneal metastases of colorectal origin was demonstrated in 2016.⁷¹ In this study, peritoneal metastases from non-mucinous adenocarcinoma were accurately identified following the intravenous administration of free ICG during surgery, leading to an adjustment in clinical decision making in 29% of patients. However, the benefit was minimal in patients with mucinous adenocarcinoma. Despite the positive results demonstrated in this study, the main disadvantage still lies in the fact it is not tumor-specific and therefore leads to a low sensitivity and specificity.

5.2 Targeted fluorescent probes

Due to the low sensitivity and specificity of non-targeted fluorescence probes, its application in surgical oncology is still limited. Therefore, to increase contrast, this resulted in a shift toward the development and clinical translation of targeted optical imaging agents enhancing surgical vision based on the molecular characteristics of cancer cells.

The MOA of targeted fluorescent probes is based on the concept of a carrier molecule that is conjugated to a fluorescent dye, specifically binding to a certain tumor target. Carrier molecules can either be monoclonal antibodies, (small) peptides, small molecules, or other molecules that specifically target certain cell surface markers that become overexpressed due to genetic variances that occur in every tumor (Figure 3B).⁷² Moreover, the increased metabolic activity that characterizes certain tumor types can be used as a target.⁴⁰

Besides a suitable carrier molecule, the fluorescent dye itself also plays an important role. The development of new fluorescence probes is challenging since each agent needs separate regulatory approval, which is an expensive and time-consuming process.⁷³ As mentioned before, fluorescent dyes that emit light in the NIR light spectrum provide several advantages over dyes that emit light in the visible light spectrum. Although there is a wide variety of fluorescent dyes available for conjugation to carrier molecules, the most preclinical and clinical experience has been obtained with the NIR fluorescent dye IRDye800CW, developed by LI-COR Biosciences Inc. (Lincoln, NE). The IRDye800CW has a peak emission wavelength at 794 nm and is ideal for protein and antibody labeling, as conjugation to a carrier molecule is relatively easy and extensive toxicity studies have been performed.⁷⁴

The increasing clinical application of therapeutic monoclonal antibodies specifically targeting certain biomarkers of cancer is an interesting development in the perspective of optical molecular imaging. Targeting certain tumor-specific receptors with fluorescently labeled antibodies seems to have great potential for visualization of cancer during interventions, also in CRC.^{72, 75-78} Multiple targeted probes have already been tested successfully in several preclinical studies.^{75, 77-84}

The first in-human proof-of-principle of targeted optical molecular imaging using a fluorescent probe was provided by van Dam et al in 2011, demonstrating the potential of MFGS in patients with PC originating from ovarian cancer using the fluorescent tracer folate- FITC, targeting the folate receptor α .²⁴ Ever since, targeted optical imaging has been applied for many different indications.

6. POTENTIAL FLUORESCENCE IMAGING AGENTS FOR DETECTION OF COLORECTAL CANCER

As mentioned before, using the TASC scoring system, seven potential targets for optical molecular imaging of PC of colorectal origin have been identified: CXCR4, EpCAM, EGFR, CEA, Muc1, MMPs, and VEGF-A.⁴⁶ The specifics of these proteins and receptors are summarized in Table 1.⁸⁵⁻¹⁰⁹ Several fluorescent imaging probes targeting these biomarkers have already been investigated in humans in a broad variety of indications.

For example, the NIR fluorescent tracer cetuximab-800CW targeting EGFR has been applied in humans for surgical navigation in head-and-neck squamous cell carcinoma.⁹²

Table 1. Potential targets for optical molecular imaging in PC of colorectal origin using the TASC scoring system.

Target	Name	Location	Function	Over-expression in CRC	Carrier molecule clinically available	GMP-labeled fluorescent tracer	Clinical trials in humans
CXCR4	Chemokine Receptor 4	Cell surface	Homing of hematopoietic stem cells to the bone-marrow	±70% ⁸⁵	AMD3100 (molecule) ⁸⁶ SDF-1a (peptide) ⁸⁶ 12G5 (mAb) ⁸⁶	-	-
EpCAM	Epithelial Cell Adhesion Molecule	Cell surface	Cell adhesion	>80% ^{87,88}	Edrecolomab Catumaxomab	323/A3-800CW ⁸⁹	-
EGFR	Epidermal Growth Factor Receptor	Cell surface	Cell proliferation, differentiation, adhesion and migration	±80% ^{90,91}	Cetuximab Panitumumab	Cetuximab-800CW Panitumumab-800CW	NCT03134846 ⁹²
CEA	Carcino-embryogenic antigen	Cell surface	Cell adhesion	>90% ⁹³⁻⁹⁵	Arcitumomab	SGM-101 ⁹⁶	-
Muc1	Mucin-1	Cell surface	Forming protective mucous barriers on epithelial surfaces, intracellular signaling (cell adhesion and anti-adhesion)	±50% ^{97,98}	Muc1-targeting peptide C595 (mAb) Bispecific anti-Muc1 antibody	-	-
MMP	Matrix Metalloproteinases	Tumor micro-environment	Degrading proteins in extracellular matrix	30-95% depending on the type ^{97,99-102}	-	-	-
VEGF-A	Vascular Endothelial Growth Factor-A	Tumor micro-environment	Angiogenesis	Up to 96% ^{103,104}	Bevacizumab	Bevacizumab-800CW ¹⁰⁵	NCT02113202 NCT01972373 NCT02583568 NCT02975219 NCT02743975 NCT01691391 ¹⁰⁶⁻¹⁰⁹

Moreover, cetuximab-800CW is being used in a phase-I clinical trial in the University Medical Center Groningen in patients with head and neck squamous cell carcinoma (NCT03134846).

Besides, the NIR fluorescent tracer bevacizumab-800CW targeting VEGF-A has been applied for detection of a variety of different tumor types, among which locally advanced rectal cancer (NCT01972373), pancreatic cancer (NCT02743975), breast cancer¹⁰⁹ (NCT02583568), and esophageal cancer.¹⁰⁸ The feasibility of MFGS using bevacizumab-800CW is also being investigated for intraoperative guidance in benign diseases such as endometriosis (NCT02975219) or for endoscopic detection of familial adenomatous polyposis (NCT02113202).

In CRC and specifically peritoneal metastases of colorectal origin, so far two phase-I feasibility studies have been performed in humans.

7. FEASIBILITY STUDIES IN PC OF COLORECTAL ORIGIN

In 2016, Harlaar et al used the NIR fluorescent tracer bevacizumab-RDye800CW targeting VEGF-A for MFGS in seven patients with PC from CRC origin, that were scheduled to undergo CRS and HIPEC.¹⁰⁷ Intravenous administration of bevacizumab-800CW three days prior to surgery proved to be safe, as no (serious) adverse events that were related to tracer administration occurred in any of the patients. Fluorescence signals were observed in all patients during surgery. Additional tumor tissue that had not been identified by the surgeons using only visual and tactile inspection, was detected in two patients using fluorescence imaging. The fresh surgical specimens were imaged back-table at the operating theatre. A total of 80 peritoneal areas were imaged using the intraoperative camera system and analyzed by a pathologist. All 29 resected, but non-fluorescent areas proved to be benign on final histopathology, thus potentially indicating a sensitivity of 100%. In 27 out of 57 fluorescent areas in the fresh surgical specimen, tumor tissue was identified. Although the authors state that their study was not powered to investigate the sensitivity and specificity, the results are very promising. In conclusion, in this study MFGS using bevacizumab-800CW was safe and feasible and could potentially improve CRS and patient selection.

The second feasibility study was performed in 2018 by Boogerd et al, in which SGM-101, a fluorescent anti-CEA monoclonal antibody, was administered intravenously 2–4 days before surgery, to investigate the feasibility of MFGS in CRC and PC of colorectal origin.⁹⁶ Patients with PC of colorectal origin that were scheduled for open surgical removal were included. First, a dose-finding study was performed in the first nine patients. Subsequently, the most optimal dose of SGM-101 was investigated in another 17 patients. SGM-101 showed no treatment-related (serious) adverse events. However, a total of eight possibly related mild adverse events occurred throughout the study. Using MFGS, in six patients a total of 19 additional peritoneal lesions were identified as potentially tumor-positive, and therefore treatment strategies were changed. The authors report a sensitivity of 98% and a specificity of 62%.

Interestingly, although both studies used different fluorescent tracers, more or less the same conclusions were drawn. Most importantly, both bevacizumab-800CW and SGM-101 were deemed safe in combination with MFGS. Moreover, it appeared that with both fluorescent tracers, a very high sensitivity could be obtained. If these results are validated in a larger patient cohort and indeed clinically suspect, but non-fluorescent lesions turn out to be benign, non-fluorescent lesions may be left in situ in the future and subsequently decrease morbidity. Interestingly, this might also imply that currently visual and tactile inspection-based surgery leads to unnecessary resections when compared to MFGS. The majority of complications and revalidation time is probably related to the extent of the cytoreduction itself. This might also improve current morbidity of 22-34% and mortality of 0.8-4.1%.¹¹⁰⁻¹¹⁵

The specificity in these two feasibility studies appears to be relatively low, with a substantial amount of false positive lesions when applying intraoperative fluorescence imaging. This might be due to technical limitations of the fluorescence camera system that still need to be improved, such as the multispectral subtraction techniques. Currently, quantification of fluorescence with most of the present generation of clinically approved fluorescence camera systems is still limited, making the interpretation of fluorescence signals subjective. If a threshold could be set to give the surgeon a “yes” or “no” answer to the question whether a peritoneal lesion is tumor-positive with a certain sensitivity and specificity, this could potentially improve interpretation of fluorescence signals. Last, for some tracers, the optimal dose might still need further optimization.¹⁰⁷

Additional research and studies need to be performed to investigate novel fluorescence imaging agents in humans for MFGS of PC of colorectal origin. Theoretically, multiple fluorescence imaging agents can be intravenously administered to the same patients simultaneously, a so-called “tracer cocktail,” in order to improve sensitivity and specificity. Therefore, novel fluorescence imaging agents need to be validated in a standardized way, with the emphasis on the determination of the safety, feasibility, optimal agent dose, and optimal timing for surgical intervention in phase-I feasibility studies.

8. FUTURE PERSPECTIVES

8.1 Target selection for MFGS

Currently, there are many carrier molecules that seem promising for potential validation in phase-I feasibility studies according to the TASC scoring system.⁴⁶ Additionally, new strategies have been developed recently to identify biomarkers that are upregulated in cancer development, such as functional genomic mRNA (FGM) profiling.¹¹⁶ This method corrects expression data of numerous genes for relevant non-genetic variables. It is likely that in the near future new promising targets will be identified by this gene expression analysis, that may be used as targets, providing new possibilities for imaging of PC of colorectal origin.

8.2 Novel fluorescence imaging probes

Next to the validation of potential targets for imaging, novel fluorescent probes are being developed.^{72,117} Different types of carrier molecules have different pharmacokinetics. The substantial molecular weight of monoclonal antibodies (generally $\pm$ 150 kDa, Figure 3B) results in a relatively long blood circulation time of several days up to weeks. Although there is extensive experience with the use of monoclonal antibodies, smaller molecules may provide favorable pharmacokinetic properties, such as nanobodies.¹¹⁸ A faster clearance from background tissue results in sufficient signal-to-background ratios that occur within a much shorter period of time. Therefore, peptides or small molecules might be logistically favorable compared to antibodies for MFGS (Figures 3C and D).¹¹⁸ On the other hand, smaller molecules are in general more difficult to conjugate to a fluorescence dye, as even small structural changes can influence pharmacokinetics and binding efficacy significantly.^{118,119}

Another subgroup of imaging probes has come forward in recent years: targeted smart-activatable probes (Figure 3E).¹¹⁹ The working mechanism of these probes is based on the principle of photochemical quenching or ligand-targeted activation. Smart activatable probes only fluoresce when bound to the tumor or cleaved by specific proteases or peptidases excreted by the tumor, which improves signal-to-background ratios due to limited background fluorescence.^{118,120} The first clinical studies to investigate smart activatable probes have been performed already.¹¹⁷ However, to the best of our knowledge, this has not yet been done for intraoperative imaging of CRC or PC of colorectal origin.

A similar “on-or-off” concept has been applied in the development of a pH-activatable fluorescent probe. This probe becomes activated upon contact with a certain threshold pH ($\text{pH} \leq 6.9$), as the majority of solid tumors are acidotic. Although this probe does not target a tumor biomarker, it is still highly specific due to the pH transistor concept.¹²¹ The benefit of such a probe is that it can be applied in a broad range of oncological indications. However, the first proof-of-concept in human study using a pH-activated probe still needs to be conducted.

8.3 Phase II/III clinical studies

Although different fluorescent probes are being developed, so far only two phase-I feasibility studies have been finalized in relatively small numbers of patients with PC of colorectal origin.^{96, 107} The ability of fluorescence imaging to detect peritoneal metastases that are missed by visual and tactile inspection and to aid in the differentiation between malignant and benign tissue, may have the potential to change clinical decision making. Although these results seem promising, further validation in phase-II clinical studies is required, with larger patient cohorts that are sufficiently powered to estimate the diagnostic accuracy. Eventually, in phase-III studies, the impact of MFGS in CRS and HIPEC surgery on clinical endpoints such as progression-free and overall survival need to be evaluated, hoping to improve the current median progression-free survival of only 12.6 months.¹¹

8.4 Photodynamic therapy

Although current clinical studies are mainly aimed to investigate the feasibility of optical imaging for cancer detection, in the future intraoperative imaging may also be used as a therapeutic modality. Carrier molecules that specifically target the tumor can also be labeled to a photoactive dye (i.e., photosensitizer), to allow targeted photodynamic therapy (tPDT). When excited with light of a specific wavelength, photosensitizers not only fluoresce, but also form reactive oxygen species that oxidize the cells they target, thereby killing them.¹²² Potentially, tPDT may be applied after CRS, to assist in the elimination of microscopic peritoneal lesions. As there is only superficial activation of the targeted photosensitizers, side-effects are estimated to be limited. The first clinical trials have already been performed to investigate the safety and feasibility of tPDT using a variety of different photosensitizers. Phase I and II clinical trials have been conducted for treatment of colorectal cancer,¹²³ pelvic recurrence of CRC,¹²⁴ colorectal liver metastases (NCT00068068),¹²⁵ and locally advanced rectal cancer.¹²⁶ Moreover, tPDT has also been applied for the treatment of peritoneal metastases originating from ovarian cancer and sarcomas,¹²⁷ and different gastrointestinal tumors¹²⁸ with promising results. These studies demonstrate that tPDT could potentially be used as an effective treatment for both CRC and PC. However, future studies are required to determine the effect on PC of colorectal origin, when combined with MFGS.

9. CONCLUSION

In conclusion, treatment of PC of colorectal origin with curative intent consists of CRS followed by HIPEC. Up to date, surgeons still rely on visual and tactile inspection for intraoperative differentiation between tumor and benign tissue. The ultimate goal during cytoreduction is to obtain a macroscopically complete cytoreduction by resecting malignant tissue only. Therefore, there is a clear need for an intraoperative imaging technique improving tumor detection. The first phase-I clinical trials have been performed showing the potential benefit of MFGS for patients with PC of colorectal origin. Even though no conclusions can be drawn with regard to the impact of these studies on clinical decision making, it appears MFGS has the potential to improve both cytoreduction and patient selection, facilitating patient- tailored surgery. However, to reliably determine the sensitivity and specificity of MFGS during CRS and HIPEC, subsequent phase-II studies are required.

CONFLICTS OF INTEREST

None of the authors have declared any conflicts of interest.

SYNOPSIS

This paper gives a chronological overview in the development of Molecular Fluorescence Guided Surgery during Cytoreductive Surgery and HIPEC treatment in patients with Peritoneal Carcinomatosis of colorectal origin.

REFERENCES

- 1 Torre LA, Bray F, Siegel RL, et al. Global cancer statistics, 2012. *CA Cancer J Clin.* 2015;65:87-108.
- 2 Siegel RL, Miller KD, Fedewa SA, et al. Colorectal cancer statistics, 2017. *Ca Cancer J Clin.* 2017;67:177-193.
- 3 Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin.* 2005;55:74-108.
- 4 Koppe MJ, Boerman OC, Oyen WJ, Bleichrodt RP. Peritoneal carcinomatosis of colorectal origin: incidence and current treatment strategies *Ann surg.* 2006;243:212-222.
- 5 Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. *CA Cancer J Clin.* 2013;63:11-30.
- 6 Segelman J, Granath F, Holm T, et al. Incidence, prevalence and risk factors for peritoneal carcinomatosis from colorectal cancer. *Br J Surg.* 2012;99:699-705.
- 7 Yan TD, Chu F, Links M, et al. Cytoreductive surgery and perioperative intraperitoneal chemotherapy for peritoneal carcinomatosis from colorectal carcinoma: non-mucinous tumour associated with an improved survival. *Eur J Surg Oncol.* 2006;32:1119-1124.
- 8 Sugarbaker PH. A curative approach to peritoneal carcinomatosis from colorectal cancer. *Semin Oncol.* 2005;32:S68-S73.
- 9 Sugarbaker PH. Peritonectomy procedures. *Cancer Treat Res.* 2007;134:247-264.
- 10 Sugarbaker PH. Peritonectomy procedures. *Ann Surg.* 1995;221:29-42.
- 11 Verwaal VJ, Bruin S, Boot H, et al. 8-year follow up of randomized trial: cytoreduction and hyperthermic intraperitoneal chemotherapy versus systematic chemotherapy in patients with peritoneal carcinomatosis of colorectal cancer. *Ann surg Oncol.* 2008;15:2426-2432.
- 12 Verwaal VJ, van Ruth S, de Bree E, et al. Randomized trial of cytoreduction and hyperthermic intraperitoneal chemotherapy versus systemic chemotherapy and palliative surgery in patients with peritoneal carcinomatosis of colorectal cancer. *J Clin Oncol.* 2003;21:3737-3743.
- 13 Elias D, Gilly F, Boutitie F, et al. Peritoneal colorectal carcinomatosis treated with surgery and perioperative intraperitoneal chemotherapy: retrospective analysis of 523 patients from a multicentric French Study. *J Clin Oncol.* 2010;28:63-68.
- 14 Sugarbaker PH, Ryan DP. Cytoreductive surgery plus hyperthermic perioperative chemotherapy to treat peritoneal metastases from colorectal cancer: standard of care or an experimental approach? *Lancet Oncol.* 2012;13:e362-e369.
- 15 Shan LL, Saxena A, Shan BL, Morris DLI. Quality of life after cytoreductive surgery and hyperthermic intra-peritoneal chemotherapy for peritoneal carcinomatosis: a systematic review and meta-analysis. *Surg Oncol.* 2014;23:199-210.
- 16 Braam HJ, van Oudheusden TR, de Hingh IH, et al. Patterns of recurrence following complete cytoreductive surgery and hyperthermic intraperitoneal chemotherapy in patients with peritoneal carcinomatosis of colorectal cancer. *J Surg Oncol.* 2014;109:841-847.
- 17 Klumpp BD, Schwenzer N, Aschoff P, et al. Preoperative assessment of peritoneal carcinomatosis: intraindividual comparison of 18F-FDG PET/CT and MRI. *Abdom Imaging* 2013;38:64-71.
- 18 De Bree E, Koops W, Kröger R, et al. Peritoneal carcinomatosis from colorectal or appendiceal origin: correlation of preoperative CT with intraoperative findings and evaluation of interobserver agreement. *J Surg Oncol.* 2004;86:64-73.
- 19 Dromain C, Leboulleux S, Auperin A, et al. Staging of peritoneal carcinomatosis: enhanced CT vs PET/CT. *Abdom Imaging.* 2008;33:87-89.
- 20 Jacquet P, Jelinek JS, Steves MA, Sugarbaker PH. Evaluation of computed tomography in patients with peritoneal carcinomatosis. *Cancer* 1993;72:1631-1636.
- 21 Coakley FV, Choi PH, Gougoutas CA, et al. Peritoneal metastases: detection with spiral CT in patients with ovarian cancer. *Radiology* 2002;223:495-499.
- 22 González-Moreno S, González-Bayón L, Ortega-Pérez, González-Hernando. Imaging of peritoneal carcinomatosis. *Cancer J.* 2009;15:184-189.
- 23 Low RN, Barone RM, Lucero J. Comparison of MRI and CT for predicting the Peritoneal Cancer Index (PCI) preoperatively in patients being considered for cytoreductive surgical procedures. *Ann Surg Oncol.* 2015;22:1708-1715.

- 24 van Dam GM, Themelis G, Crane LM, et al. Intraoperative tumor-specific fluorescence imaging in ovarian cancer by folate receptor- α targeting: first in-human results. *Nat. Med.* 2011;17:1315-1319.
- 25 Tummers QR, Hoogstins CE, Gaarenstroom KN, et al. Intraoperative imaging of folate receptor alpha positive ovarian and breast cancer using the tumor specific agent EC17. *Oncotarget* 2016;7:32144-32155.
- 26 de Boer E, Warram JM, Tucker MD, et al. In vivo fluorescence immunohistochemistry: localization of fluorescently labeled cetuximab in squamous cell carcinomas. *Sci Rep.* 2015;5:10169.
- 27 de Boer E, Harlaar NJ, Taruttis A, et al. Optical innovations in surgery. *Br J Surg.* 2015;102:e56-e72.
- 28 Weissleder R, Ntziachristos V. Shedding light into 137.live molecular targets. *Nat Med.* 2003;9:123-128.
- 29 Frangioni JV. In vivo near-infrared fluorescence imaging. *Curr Opin Chem Biol.* 2003;7:626-634.
- 30 Hilderbrand SA, Weissleder R. Near-infrared fluorescence: application to in vivo molecular imaging. *Curr Opin in Chem Biol* 2010;14:71-79.
- 31 de Grand AM, Frangioni JV. An operational near-infrared fluorescence imaging system prototype for large animal surgery. *Technol Cancer Res Treat.* 2003;2:553-562.
- 32 Nakayama A, del Monte F, Hajjar RJ, Frangioni JV. Functional near-infrared fluorescence imaging for cardiac surgery and targeted gene therapy. *Mol Imaging.* 2002;1:365-377.
- 33 Ntziachristos V, Bremer C, Weissleder R. Fluorescence imaging with near-infrared light: new technological advances that enable in vivo molecular imaging. *Eur Radiol.* 2003;13:195-208.
- 34 Tanaka E, Choi HS, Fuji H, et al. Image-guided oncologic surgery using invisible light: completed pre-clinical development for sentinel lymph node mapping. *Ann Surg Oncol.* 2006;13:1671-1681.
- 35 Themelis G, Yoo JS, Soh KS, et al. Real-time intraoperative fluorescence imaging system using light-absorption correction. *J Biomed Opt.* 2009;14:064012.
- 26 Troyan SL, Kianzad V, Gibbs-Strauss SL, et al. The FLARE intraoperative near-infrared fluorescence imaging system: a first-in-human clinical trial in breast cancer sentinel lymph node mapping. *Ann Surg Oncol.* 2009;16:2943-2952.
- 37 Glatz J, Varga J, Garcia-Allende PB, et al. Concurrent video-rate color and near-infrared fluorescence laparoscopy. *J Biomed Opt.* 2013;18:10132.
- 38 Garcia-Allende PB, Glatz J, Koch M, et al. Towards clinically translatable NIR fluorescence molecular guidance for colonoscopy. *Biomed Opt Express.* 2013;5:78-92.
- 39 Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100:57-70.
- 40 Keereweer S, Kerrebijn JD, van Driel PB, et al. Optical image-guided surgery – where do we stand? *Mol Imaging Biol.* 2011;13:199-207.
- 41 Kroemer G, Pouyssegur J. Tumor cell metabolism: cancer's Achilles' heel. *Cancer cell* 2008;13:472-482.
- 42 Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011;144:646-674.
- 43 Day DW, Morson BC. The adenoma-carcinoma sequence. *Major Probl Pathol.* 1978;10:58-71.
- 44 Cardoso J, Boer J, Morreau H, Fodde R. Expression and genomic profiling of colorectal cancer. *Biochim Biophys Acta.* 2007;1775:103-e72.
- 45 Lin YM, Furukawa Y, Tsunoda T, et al. Molecular diagnosis of colorectal tumors by expression profiles of 50 genes expressed differentially in adenomas and carcinomas. *Oncogene* 2002;21:4120-4128.
- 46 van Oosten M, Crane LM, Bart J, et al. Selecting potential targetable biomarkers for imaging purposes in colorectal cancer using target selection criteria (TASC): a novel target identification tool. *Transl Oncol.* 2011;4:71-82.
- 47 Paumgartner G, Probst P, Kraines R, Leevy CM. Kinetics of indocyanine green removal from the blood. *Ann N Y Acad Sci.* 1970;170:134-147.
- 48 Cherrick GR, Stein SW, Leevy CM, Davidson CS. Indocyanine green: observation on its physical properties, plasma decay, and hepatic extraction. *J Clin Invest.* 1960;39:592-600.
- 49 Sakka SG. Assessing liver function. *Curr Opin Crit Care.* 2007;13:207-214.
- 50 Wada H, Hyuan H, Vargas C, et al. Sentinel lymph node mapping of liver. *Ann Surg Oncol.* 2015;22:1147-1155.
- 51 Toyota T, Fujito H, Suganami Am et al. Near-infrared-fluorescence imaging of lymph nodes by using liposomally formulated indocyanine green derivatives. *Bioorg Med Chem.* 2014;22:721-727.

- 52 Paulinelli RR, Freitas-Junior R, Rahal RM, et al. A prospective randomized trial comparing patent blue and methylene blue for the detection of the sentinel lymph node in breast cancer patients. *Rev Assoc Med Bras.* 1992;63:118-123.
- 53 Fattahi AS, Tavassoli A, Rohbakhshfar O, et al. Can methylene blue dye be used as an alternative to patent blue dye to find the sentinel lymph node in breast cancer surgery? *J Res Med Sci.* 2014;19:918-922.
- 54 Landsman ML, Kwant G, Mook GA, Zijlstra WG. Light-absorbing properties, stability, and spectral stabilization of indocyanine green. *J Appl Physiol.* 1976;40:575-583.
- 55 Muckle TJ. Plasma proteins binding of indocyanine green. *Biochem Med.* 1976;15:17-
- 56 Mothes H, Döncke T, Friedel R, et al. Indocyanine-green fluorescence video angiography used clinically to evaluate tissue perfusion in microsurgery. *J Trauma.* 2004;57:1018-1024.
- 57 Leevy CM, Smith F, Longueville J, et al. Indocyanine green clearance as a test for hepatic function. Evaluation by dichromatic ear densitometry. *JAMA.* 1967;200:236-240.
- 58 Reuthebuch O, Häussler A, Genoni M, et al. Novadaq SPY: intraoperative quality assessment in off-pump coronary artery bypass grafting. *Chest* 2004;125:418-424.
- 59 Desai N, Fremes SE. Intraoperative indocyanine green angiography: ready for prime time? *J Thorac Cardiovasc Surg.* 2007;133:1396-1397.
- 60 Desai ND, Miwa S, Kodama D, et al. A randomized comparison of intraoperative indocyanine green angiography and transit-time flow measurement to detect technical errors in coronary bypass grafts. *J Thorac Cardiovasc Surg.* 2006;132:585-594.
- 61 Rubens FD, Ruel M, Fremes SE. A new and simplified method for coronary and graft imaging during CABG. *Heart Surg Form.* 2002;5:141-144.
- 62 Balacumaraswami L, Abu-Omar Y, Choudhary B, et al. A comparison of transit-time flowmetry and intraoperative fluorescence imaging for assessing coronary artery bypass graft patency. *J thorac Cardiovasc Surg.* 2005;130:315-320.
- 63 Schaafsma BE, Mieog SJ, Hutteman M, et al. The clinical use of indocyanine green as a near-infrared fluorescent contrast agent for image-guided oncologic surgery. *J Surg Oncol.* 2011;104:323-332.
- 64 Ishizawa T, Fukushima N, Shibahara J, et al. Real-time identification of liver cancers by using indocyanine green fluorescent imaging. *Cancer* 2009;115:2491-2504.
- 65 Raabe A, Beck J, Gerlach R, et al. Near-infrared indocyanine green video angiography: a new method for intraoperative assessment of vascular flow. *Neurosurgery.* 2003;52:132-139.
- 66 Raabe A, Nakaji P, Beck J, et al. Prospective evaluation of surgical microscope-integrated intraoperative near-infrared indocyanine green videoangiography during aneurysm surgery. *J Neurosurg.* 2005;103:982-989.
- 67 Singh SK, Desai ND, Chikazawa G, et al. The Graft Imaging to Improve Patency (GRIIP) clinical trial results. *J Thorac Cardiovasc Surg.* 2010;139:294-301.
- 68 Zimmermann A, Roenneberg C, Wendorff H, et al. Early postoperative detection of tissue necrosis in amputation stumps with indocyanine green fluorescence angiography. *Vasc Endovascular Surg.* 2010;44:269-273.
- 69 Holm C, Mayr M, Höfner E, et al. Assessment of the patency of microvascular anastomoses using microscope-integrated near-infrared angiography: a preliminary study. *Microsurgery.* 2009;29:509-514.
- 70 Holm C, Dornseifer U, Sturtz G, Ninkovic M. Sensitivity and specificity of ICG angiography in free flap reexploration. *J Reconstr Microsurg.* 2010;26:311-316.
- 71 Liberale G, Vankerckhove S, Caldon MG, et al. Fluorescence imaging after indocyanine green injection for detection of peritoneal metastases in patients undergoing cytoreductive surgery for peritoneal carcinomatosis from colorectal cancer: a pilot study. *Ann Surg.* 2016;264:1110-1115.
- 72 Zhang RR, Schroeder AB, Grudzinski JJ, et al. Beyond the margins: real-time detection of cancer using targeted fluorophores. *Nat Rev Clin Oncol.* 2017;14:347-364.
- 73 Verbeek FPR, van der Vorst JR, Schaafsma BE, et al. Image-guided hepatopancreatobiliary surgery using near-infrared fluorescent light. *J Hepatobiliary Pancreat Sci.* 2012;19:626-637.
- 74 Marshall MV, Draney D, Sevcik-Muraca EM, Olive DM. Single-dose intravenous toxicity study of IRDye 800CW in Sprague-Dawley rats. *Mol Imaging Biol.* 2010;12:583-594.

- 75 Day KE, Sweeny L, Kulbersh B, et al. Preclinical comparison of near-infrared-labeled cetuximab and panitumumab for optical imaging of head and neck squamous cell carcinoma. *Mol Imaging Biol.* 2013;15:722-729.
- 76 Stummer W, Reulen HJ, Novotny A, et al. Fluorescence-guided resections of malignant gliomas – an overview. *Acta Neurochir Suppl.* 2003;88:9-12.
- 77 Heath CH, Deep NL, Sweeny L, et al. Use of panitumumab-IRDye800 to image microscopic head and neck cancer in an orthotopic surgical model. *Ann Surg Oncol.* 2012;19:3879-3887.
- 78 Terwisscha van Scheltinga AG, van Dam GM, Nagengast WB, et al. Intraoperative near-infrared fluorescence tumor imaging with vascular endothelial growth factor and human epidermal growth factor receptor 2 targeting antibodies. *J Nucl Med.* 2011;52:1778-1785.
- 79 Mieog JS, Vahrmeijer AL, Hutteman M, et al. Novel intraoperative near-infrared fluorescence camera system for optical image-guided cancer surgery. *Mol Imaging* 2010;9:223-231.
- 80 Mieog JS, Hutteman M, Vorst JR, et al. Image-guided tumor resection using real-time near-infrared fluorescence in a syngeneic rat model of primary breast cancer. *Breast Cancer Res Treat.* 2011;128:679-689.
- 81 Hutteman M, Mieog JS, van der Vorst JR, et al. Intraoperative near-infrared fluorescence imaging of colorectal metastases targeting integrin $\alpha(v)\beta(3)$ expression in a syngeneic rat model. *Eur J Surg Oncol.* 2011;37:252-257.
- 82 Adams KE, Ke S, Kwon S, et al. Comparison of visible and near-infrared wavelength-excitable fluorescent dyes for molecular imaging of cancer. *J Biomed Opt.* 2007;12:024017.
- 83 Weissleder R, Tung CH, Mahmood U, Bogdanov A Jr. In vivo imaging of tumors with protease-activated near-infrared fluorescent probes. *Nat Biotechnol.* 1999;17:375-378.
- 84 Korb ML, Hartman YE, Kovar J, et al. Use of monoclonal antibody-IRDye800CW bioconjugates in the resection of breast cancer. *J Surg Res.* 2014;188:119-128.
- 85 Rubie C, Kollmar O, Frick VO, et al. Differential CXCR4 receptor expression in colorectal carcinomas. *Scand J Immunol.* 2008;68:635-644.
- 86 Nayak TR, Hong H, Zhang Y, Cai W. Multimodality imaging of CXCR4 in cancer: current status towards clinical translation. *Curr Mol Med.* 2013;13:1538-1548.
- 87 Paret C, Hildebrand D, Weitz J, et al. C4.4A as a candidate marker in the diagnosis of colorectal cancer. *Br J Cancer.* 2007;97:1146-1156.
- 88 Xie X, Wang CY, Cao YX, et al. Expression pattern of epithelial cell adhesion molecule on normal and malignant colon tissues. *World J Gastroenterol.* 2005;11:344-347.
- 89 Van Driel PBAA, Boonstra MC, Prevoo HAJM, et al. EpCAM as multi-tumour target for near-infrared fluorescence guided surgery. *BMC Cancer* 2016;12:884.
- 90 Cunningham MP, Essapen S, Thomas H, et al. Coexpression of the IGF-IR, EGFR and HER-2 is common in colorectal cancer patients. *Int J Oncol.* 2006;16:329-335.
- 91 Goldstein NS, Armin M. Epidermal growth factor receptor immune-histochemical reactivity in patients with American Joint Committee on Cancer Stage IV colon adenocarcinoma: implications for a standardized scoring system. *Cancer* 2001;92:1331-1346.
- 92 Rosenthal EL, Warram JM, de Boer E, et al. Safety and tumor specificity of cetuximab-IRDye800 for surgical navigation in head and neck cancer. *Clin Cancer Res.* 2015;21:3658-3666.
- 93 Li M, Li JY, Zhao AL, et al. Comparison of carcinoembryonic antigen prognostic value in serum and tumour tissue of patients with colorectal cancer. *Colorectal Dis.* 2009;11:276-281.
- 94 Kim JC, Gong G, Roh SA, Park KC. Carcinoembryonic antigen gene and carcinoembryonic antigen expression in the liver metastasis of colorectal carcinoma. *Mol Cells* 1999;9:133-137.
- 95 Suwanagool P, Fujimori T, Maeda S. Value of tissue carcinoembryonic antigen in patients with colorectal carcinoma. *Asian Pac J Allergy Immunol.* 1990;8:33-37.
- 96 Boogerd LSF, Hoogstins CES, Schaap DP, et al. Safety and effectiveness of SGM-101, a fluorescent antibody targeting carcinoembryonic antigen, for intraoperative detection of colorectal cancer: a dose-escalation pilot study. *Lancet Gastroenterol Hepatol.* 2018;3:181-191.
- 97 Kaneko I, Tanaka S, Oka S, et al. Immunohistochemical molecular markers as predictors of curability of endoscopically resected submucosal colorectal cancer. *World J Gastroenterol.* 2007;13:3829-3835.

- 98 Suzuki H, Shoda J, Kawamoto T, et al. Expression of MUC1 recognized by monoclonal antibody MY .1E12 is a useful biomarker for tumor aggressiveness of advanced colon carcinoma. *Clin Exp Metastasis* 2004;21:321–329.
- 99 McKerrow JH, Bhargava V, Hansell E, et al. A functional proteomics screen of proteases in colorectal carcinoma. *Mol Med.* 2000;6:450–460.
- 100 Jeffery N, McLean MH, El Omar EM, Murray GI. The matrix metalloproteinase/tissue inhibitor of matrix metalloproteinase profile in colorectal polyp cancers. *Histopathology* 2009;54:820–828.
- 101 Madoz-Gúrpide J, López-Serra P , Martínez-Torrecaudrada JL, et al. Proteomics-based validation of genomic data: applications in colorectal cancer diagnosis. *Mol Cell Proteomics* 2006;5:1471–1483.
- 102 Emmert-Buck MR, Roth MJ, Zhuang Z, et al. Increased gelatinase A (MMP-2) and cathepsin B activity in invasive tumor regions of human colon cancer samples. *Am J Pathol.* 1994;145:1285–1290.
- 103 Cao D, Hou M, Guan YS, et al. Expression of HIF-1 α and VEGF in colorectal cancer: association with clinical outcomes and prognostic implications. *BMC Cancer* 2009;9:432.
- 104 Abdou AG, Aiad H, Asaad N, et al. Immunohistochemical evaluation of vascular endothelial growth factor (VEGF) in colorectal carcinoma. *J Egypt Natl Canc Inst.* 2006;18:311–322.
- 105 Ter Weele EJ, Terwisscha van Scheltinga AG, Linssen MD, et al. Development, preclinical safety, formulation, and stability of clinical grade bevacizumab-800CW, a new near infrared fluorescent imaging agent for first in human use. *Eur J Pharm Biopharm* 2016;104:226–34.
- 106 Tjalma, JJ, Garcia-Allende PB, Hartmans E, et al. Molecular fluorescence endoscopy targeting vascular endothelial growth factor A for improved colorectal polyp detection. *J Nucl Med.* 2016;57:480–485.
- 107 Harlaar NJ, Koller M, de Jongh SJ, et al. Molecular fluorescence-guided surgery of peritoneal carcinomatosis of colorectal origin: a single-centre feasibility study. *Lancet Gastroenterol Hepatol.* 2016;1:283–290.
- 108 Nagengast WB, Hartmans E, Garcia-Allende PB, et al. Near-infrared fluorescence molecular endoscopy detects dysplastic oesophageal lesions using topical and systemic tracer of vascular endothelial growth factor A. *Gut.* 2017;0:1–4.
- 109 Lamberts LE, Koch M, de Jong JS, et al. Tumor-specific uptake of fluorescent bevacizumab-IRDye800CW microdosing in patients with primary breast cancer: a phase I feasibility study. *Clin Cancer Res.* 2017;23:2730–2741.
- 110 Yan TD, Deraco M, Baratti D, et al. Cytoreductive surgery and hyperthermic intraperitoneal chemotherapy for malignant peritoneal mesothelioma: multi-institutional experience. *J Clin Oncol.* 2009;27:6237–6242.
- 111 Glehen O, Gilly FN, Boutitie F, et al. Toward curative treatment of peritoneal carcinomatosis from nonovarian origin by cytoreductive surgery combined with perioperative intraperitoneal chemotherapy: a multi-institutional study of 1,290 patients. *Cancer.* 2010;116:5608–5618.
- 112 Chua TC, Moran BJ, Sugarbaker PH, et al. Early- and long-term outcome data of patients with pseudomyxoma peritonei from appendiceal origin treated by a strategy of cytoreductive surgery and hyperthermic intraperitoneal chemotherapy. *J Clin Oncol* 2012;30:2449–2456.
- 113 Bakrin N, Bereder JM, Decullier E, et al. Peritoneal carcinomatosis treated with cytoreductive surgery and Hyperthermic Intraperitoneal Chemotherapy (HIPEC) for advanced ovarian carcinoma: a French multicentre retrospective cohort study of 566 patients. *Eur J Surg Oncol.* 2013;39:1435–1443.
- 114 Kuijpers AM, Mirck B, Aalbers AG, et al. Cytoreduction and HIPEC in the Netherlands: nationwide long-term outcome following the Dutch protocol. *Ann Surg Oncol.* 2013;20:4224– 4230.
- 115 Levine EA, Stewart JH, Shen P, et al. Intraperitoneal chemotherapy for peritoneal surface malignancy: experience with 1,000 patients. *J Am Coll Surg.* 2014;218:573–585.
- 116 Fehrmann RS, Karjalainen JM, Krajewska M, et al. Gene expression analysis identifies global gene dosage sensitivity in cancer. *Nat Genet.* 2015;47:115–125.
- 117 Garland M, Yim JJ, Bogyo M. A bright future for precision medicine: advances in fluorescent chemical probe design and their clinical application. *Cell Chem Biol.* 2016;23:122–136.
- 118 Srinivasarao M, Galliford CV, Low PS. Principles in the design of ligand-targeted cancer therapeutics and imaging agents. *Nat Rev Drug Discov.* 2015;14:203–219.

- 119 Kobayashi H, Choyke PL. Target-cancer-cell-specific activatable fluorescence imaging probes: rational design and in vivo applications. *Acc Chem Res* 2011;44:83–90.
- 120 Kobayashi H, Ogawa M, Alford R, et al. New strategies for fluorescent probe design in medical diagnostic imaging. *Chem Rev*. 2010;110:2620–2640.
- 121 Zhao T, Huang G, Li Y, et al. A transistor-like pH nanoprobe for tumour detection and image-guided surgery. *Nat Biomed Eng*. 2016; doi: 10.1038/s41551-016-0006.
- 122 Plaetzer K, Krammer B, Berlanda J, et al. Photophysics and photochemistry of photodynamic therapy: fundamental aspects. *Lasers Med Sci*. 2009;24:259–268.
- 123 Kawczyk-Krupka A, Bugaj AM, Latos W, et al. Photodynamic therapy in colorectal cancer treatment: the state of the art in clinical trials. *Photodiagnosis and Photodyn Ther*. 2015;12:545–553.
- 124 Herrera-Ornelas L, Petrelli NJ, Mittelman A, et al. Photodynamic therapy in patients with colorectal cancer. *Cancer* 1986;57:677–684.
- 125 van Duijnhoven FH, Rovers JP, Engelmann K, et al. Photodynamic therapy with 5,10,15,20-tetrakis(m-hydroxyphenyl) bacteriochlorin for colorectal liver metastases is safe and feasible: results from a Phase I study. *Ann Surg Oncol*. 2005;12:808–816.
- 126 Kashtan H, Papa MZ, Wilson BC, et al. Use of photodynamic therapy in the palliation of massive advanced rectal cancer. Phase I/II study. *Dis Colon Rectum*. 1991;34:600–605.
- 127 Sindelar WF, DeLaney TF, Tochner Z, et al. Technique of photodynamic therapy for disseminated intraperitoneal malignant neoplasms. Phase I study. *Arch Surg*. 1991;126:318–324.
- 128 Webber J, Kessel D, Fromm D. Side effects and photosensitization of human tissues after aminolevulinic acid. *J Surg Res*. 1997; 68:31–37

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Molecular fluorescence-guided surgery of peritoneal carcinomatosis of colorectal origin: A single-center feasibility study

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ABSTRACT

Background

Optimum cytoreductive surgery combined with hyperthermic intraperitoneal chemotherapy (HIPEC) is essential for the curative treatment of peritoneal carcinomatosis of colorectal origin. At present, surgeons depend on visual inspection and palpation for tumour detection. Improved detection of tumour tissue using molecular fluorescence-guided surgery could not only help attain a complete cytoreduction of metastatic lesions, but might also prevent overtreatment by avoiding resection of benign lesions.

Methods

For this non-randomised, single-centre feasibility study, we enrolled patients with colorectal peritoneal metastases scheduled for cytoreductive surgery and HIPEC. Two days before surgery, 4.5 mg of the near-infrared fluorescent tracer bevacizumab-IRDye800CW was administered intravenously. The primary objectives were to determine the safety and feasibility of molecular fluorescence-guided surgery using bevacizumab-IRDye800CW. Molecular fluorescence-guided surgery was deemed safe if no allergic or anaphylactic reactions were recorded and no serious adverse events were attributed to bevacizumab-IRDye800CW. The technique was deemed feasible if bevacizumab-IRDye800CW enabled detection of fluorescence signals intraoperatively. Secondary objectives were correlation of fluorescence with histopathology by back-table imaging of the fresh surgical specimen and semi-quantitative *ex-vivo* analyses of formalin-fixed paraffin embedded (FFPE) tissue on all peritoneal lesions. Additionally, VEGF- α staining and fluorescence microscopy was done. This study is registered with the Netherlands Trial Registry, number NTR4632.

Findings

Between July 3, 2014, and March 2, 2015, seven patients were enrolled in the study. One patient developed an abdominal sepsis five days postoperatively and another died from an asystole four days postoperatively, most probably due to a cardiovascular thromboembolic event. However, both serious adverse events were attributed to the surgical cytoreductive surgery and HIPEC procedure. No serious adverse events related to bevacizumab-IRDye800CW occurred in any of the patients. Intraoperatively, fluorescence was seen in all patients. In two patients, additional tumour tissue was detected by molecular fluorescence-guided surgery that was initially missed by the surgeons. During back-table imaging of fresh surgical specimens, a total of 80 areas were imaged, marked, and analysed. All of the 29 non-fluorescent areas were found to contain only benign tissue, whereas tumour tissue was detected in 27 of 51 fluorescent areas (53%). *Ex-vivo* semi-quantification of 79 FFPE peritoneal lesions showed a tumour-to-normal ratio of 6.92 (SD 2.47).

Interpretation

Molecular fluorescence-guided surgery using the near-infrared fluorescent tracer bevacizumab-IRDye800CW is safe and feasible. This technique might be of added value for the treatment of patients with colorectal peritoneal metastases through improved patient selection and optimisation of cytoreductive surgery. A subsequent multicentre phase-II trial is needed to make a definitive assessment of the diagnostic accuracy and the effect on clinical decision making of molecular fluorescence-guided surgery.

INTRODUCTION

Colorectal cancer is one of the most common and lethal cancers worldwide, causing almost 700 000 deaths annually.¹ Peritoneal carcinomatosis occurs in about 3–21% of patients with colorectal cancer and is associated with end-stage disease.² In patients with metastatic disease limited to the peritoneal surface, cytoreductive surgery in combination with hyperthermic intraperitoneal chemotherapy (HIPEC) is deemed to be the only potentially curative treatment. This extensive procedure has a 5-year survival of up to 40% when a complete cytoreduction is achieved.³ Patients who might benefit from cytoreductive surgery and HIPEC have to be selected carefully because the procedure has a complication rate of up to 62% with a 30-day mortality of up to 7.7%.⁴ The most important prognostic factors that affect the outcome of cytoreductive surgery and HIPEC are the extent of the disease and the completeness of cytoreduction.⁵

At present, the determination of the extent of the disease is dependent on preoperative and intraoperative detection of peritoneal metastases. Current preoperative imaging modalities like CT and MRI are unable to detect small tumour deposits, mainly because of their limited resolution of 0.8 mm to 1 cm, leading to inaccurate staging.⁶ Intraoperatively, the surgeon is still dependent on visual inspection and palpation to differentiate between benign and tumour lesions, with an unknown diagnostic accuracy. Consequently, clinically suspicious but benign lesions are unnecessarily resected and small tumour lesions, which are easily missed, might be left behind.

During the past decade, molecular fluorescence-guided surgery has emerged as a promising technique for intraoperative tumour detection and margin assessment in oncological surgery.^{7,8} This technique provides real-time and tumour-specific feedback to the surgeon, thereby potentially enhancing surgical vision for the detection of small tumour deposits and enabling differentiation between benign and malignant tissue during surgery.^{8,9}

A promising target for molecular fluorescence-guided surgery in colorectal cancer is VEGF-A, an angiogenic growth factor, which is often involved in tumour-induced angiogenesis.¹⁰ VEGF-A is upregulated in 93% of the peritoneal metastasis of colorectal origin¹¹ and can be targeted by the therapeutic monoclonal antibody bevacizumab. For optical molecular imaging applications, bevacizumab has been successfully conjugated to the near-infrared fluorescent dye IRDye800CW in our current Good Manufacturing Practice facility. Bevacizumab-IRDye800CW is currently under investigation in several clinical trials for molecular fluorescence-guided surgery and for molecular fluorescence endoscopy (ClinicalTrials.gov, numbers NCT01508572, NCT01972373, NCT02129933, NCT02113202, and NCT02583568).

In this study, we determined the safety of bevacizumab-IRDye800CW and the feasibility of its use in molecular fluorescence-guided surgery for patients scheduled for cytoreductive surgery and HIPEC for the treatment of peritoneal carcinomatosis of colorectal origin.

METHODS

Study design and patients

For this non-randomised, open-label, single-arm, single-centre feasibility study, we enrolled patients who were aged 18 years or older, had histologically proven peritoneal metastases of colorectal origin, were scheduled for cytoreductive surgery and HIPEC, and had a WHO performance score of 0–2. We carefully assessed the eligibility of patients with peritoneal carcinomatosis referred for cytoreductive surgery and HIPEC in a multidisciplinary colorectal cancer meeting at the University Medical Centre Groningen (UMCG), where the study was done and eligible patients were given oral and written information about the study and the option to participate. Exclusion criteria were the presence of concomitant malignancies, distant metastasis, concurrent uncontrolled medical disorders, pregnancy or breastfeeding, clinically significant cardiac disease within the past 12 months (e.g., congestive heart failure, symptomatic coronary artery disease, and cardiac dysrhythmia or myocardial infarction), and the inability to give written informed consent.

This feasibility study was approved by the local investigational review board of the UMCG. The study was done according to the principles of the Declaration of Helsinki and the International Conference on Harmonisation of Good Medical Practices. All patients provided written informed consent before participation in the study. We appointed an independent data safety monitoring board before the start of the clinical trial. We immediately reported serious adverse events occurring during and after surgery to the investigational review board of the UMCG, the data safety monitoring board, and the Dutch Central Committee on Research Involving Human Subjects. The protocol is available online.

Procedures

Patients received 4.5 mg (1 mg/mL) of bevacizumab-IRDye800CW as an intravenous bolus injection 2 days before surgery (Supplemental figure 1). Bevacizumab-IRDye800CW was produced in the Good Manufacturing Practice facility of the UMCG, as previously described.¹²

The clinical fluorescence camera system for intraoperative use was developed by the Technical University of Munich (Helmholtz Centre, Munich) and subsequently by SurgVision BV (Explorer Air camera system, Heerenveen, Netherlands) and has been described previously by Themelis and colleagues.¹³ A sterile transparent drape (Carl Zeiss Vision BV, Oberkochen Germany) covered the camera during surgery. The imaging device was approved for intraoperative application in man by the technical department of the UMCG.

Two experienced surgical oncologists did the cytoreductive surgery and HIPEC procedures according to current clinical care, as previously described by Sugarbaker.¹⁴ After exposing the abdomen, two surgeons independently determined the clinical peritoneal cancer index (PCI) and the fluorescence PCI (fPCI) and were blinded to each other's result. To achieve blinding, one surgeon left the operating room while the other surgeon assessed first the clinical PCI by visual inspection and palpation and second the fPCI by using the intraoperative fluorescence camera system. Then the first surgeon left

the operating room and the other returned and followed the same procedure. fPCI was determined by fluorescent imaging in combination with visual inspection and palpation. After determining the PCI and fPCI, the two surgeons achieved consensus and did the cytoreductive surgery together.

After completion of the cytoreduction, the surgeons inspected the resection surface and abdominal cavity for remaining fluorescence signals using the fluorescence camera system. In case of additional fluorescence, the surgeons only took biopsy samples if they both judged the procedure to be safe for the patient. After the second intraoperative imaging, the surgeons administered HIPEC and reconstructive surgery was done according to current clinical practice.

Ex-vivo specimen analyses

To prevent possible photo bleaching, we did the handling and storage of *ex-vivo* material in a dark environment as long as this processing did not interfere with standard working procedures related to clinical care.

We did back-table fluorescence imaging of the fresh surgical specimen immediately after cytoreductive surgery in a separate room. We inspected the complete fresh surgical specimen for the presence of fluorescence signals. We aimed to collect a minimum of ten fluorescent and non-fluorescent areas per surgical specimen, which we marked with coloured pins. These areas could be either clinically suspicious for peritoneal metastases, or fluorescent lesions that were marked to learn more about the distribution and localisation of the tracer. The tissue marked by the coloured pins was histologically assessed by a pathologist who was blinded for the fluorescence signals to enable direct correlation of fluorescence signals to histopathology.

We also retrospectively assessed all peritoneal lesions to enable semi-quantification of the fluorescence signals in a reproducible way. On the basis of the pathology report, we requested, for analysis, all formalin-fixed paraffin embedded (FFPE) blocks containing malignant and benign but clinically suspicious peritoneal lesions of the included patients. If available, we also requested FFPE blocks from peritoneal metastases and clinically suspicious but benign lesions that were obtained during a previous staging laparoscopy, to serve as control tissue that contained no tracer. We scanned all FFPE blocks on the Odyssey near-infrared flatbed scanner (LI-COR Biosciences Inc, Lincoln, NE, USA), which we subsequently sliced into 10 µm thin slices. These tissue slices were mounted on silane-coated slides and dried overnight at 37°C. After the 10 µm slides had deparaffinised, we scanned all the slides with the Odyssey near-infrared flatbed scanner. Subsequently, we stained all tissue slides with haematoxylin and eosin to enable direct correlation of fluorescence signals with histopathology on the same tissue slide. A blinded and experienced pathologist segmented tumour and non-tumour areas on these haematoxylin and eosin slides, which served as a region of interest. We imported these regions of interest and the completely matching Odyssey images into the imaging analysis software ImageJ (version 1.48) to measure the mean fluorescence intensity (MFI) in benign and malignant peritoneal lesions.

We did VEGF staining on 4 µm tissue slides of all FFPE tissue blocks containing peritoneal metastases. We calculated the H-score to determine VEGF expression (Supplemental protocols).

We used fluorescence microscopy on 4 µm tissue slides to assess the tracer distribution on a microscopic level. We applied a Hoechst nucleic acid stain (33258, Invitrogen, Waltham, MA, USA) to the slides and mounted them under a cover glass in modified Kaiser's glycerine (Supplemental protocols). We used an inverted microscope (DMI6000B, Leica Biosystems GmbH, Wetzlar, Germany) for fluorescence microscopy. For optimised near-infrared visualisation, this microscope was equipped with a highly sensitive near-infrared LED light source ranging up to 900 nm (X-Cite 200DC, Excelitas Technologies, Waltham, MA, USA), an optimum near-infrared filter set (microscope two band-pass filters 850–90 m–2p and a long-pass emission filter HQ800795LP [Chroma Technology Corp, Bellows Falls, VT, USA]), a highly sensitive monochrome DFC365 FX fluorescence camera (1.4 MP CCD, Leica Biosystems GmbH), and LAS-X software (Leica Biosystems GmbH).

Outcomes

The primary objective of this study was to assess the safety and feasibility of molecular fluorescence-guided surgery using bevacizumab-IRDye800CW during cytoreductive surgery and HIPEC for the treatment of colorectal peritoneal carcinomatosis. Molecular fluorescence-guided surgery was deemed safe if no allergic or anaphylactic reactions were recorded and no serious adverse events were attributed to bevacizumab-IRDye800CW. The technique was deemed feasible if bevacizumab-IRDye800CW enabled detection of fluorescence signals intraoperatively.

The secondary objectives were correlation of fluorescence with histopathology by back-table imaging of the fresh surgical specimen and semi-quantification of all peritoneal lesions by *ex-vivo* analyses of FFPE tissue. Additionally, we aimed to determine the tracer distribution at a microscopic level.

Statistical analysis

We initially intended to include ten patients. We made no formal calculation of sample size. We did an interim analysis after five patients were recruited. The study was to be continued if at least three of five patients showed fluorescence signals in tumour tissue. Descriptive statistics were presented as a mean (SD) in case of a normal distribution, whereas median (range) was used in case of a skewed distribution. We did descriptive statistics using SPSS (version 23.0). We designed the graphs with Graphpad Prism (version 6.0). The trial was registered within the Netherlands Trial Register, number NTR4632.

Role of the funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

RESULTS

Between July 3, 2014, and March 2, 2015, seven patients were enrolled in the study. The age of the patients ranged from 26 years to 70 years (Table 1). Four patients presented with metachronous peritoneal metastases and three patients presented with synchronous peritoneal metastases (Table 1). All patients were given the tracer 2 days before receiving cytoreductive surgery and HIPEC. The planned interim analyses after recruitment of the first five patients showed fluorescence intra-operatively in all patients. However, in one patient no cancerous tissue was detected during pathological evaluation of all clinically suspicious lesions that were removed. Therefore, in four of five patients, fluorescence could be detected in tumour tissue.

No allergic or anaphylactic reactions were reported related to bevacizumab-IRDye800CW. Two serious adverse events were reported, both of which were attributed to cytoreductive surgery and HIPEC. Patient number 5 developed an abdominal sepsis at the fifth postoperative day, requiring a relaparotomy and antibiotic treatment. Patient number 7 died from an asystole 4 days after cytoreductive surgery and HIPEC, most probably due to a cardiovascular thromboembolic event. After reporting the cardiovascular thromboembolic serious adverse event, the study was put on hold. The independent investigational review board and data safety monitoring board of the UMCG did an in-depth investigation and decided that both serious adverse events were not attributable to the tracer or the imaging procedure. Because of these serious adverse events, we analysed the results of the seven patients included in the study, and did not enrol three more patients, as initially planned (Supplemental material, p1).

Table 1. Demographic and clinical data. *Mean of the scores of the two surgeons; determined by visual inspection and palpation. **Mean of the scores of the two surgeons; determined by molecular imaging. †Double tumour. ‡Signet cell tumour. ¶Curative treatment of solitary liver metastasis at primary presentation. Y = years; PC = peritoneal carcinomatosis; PCI = peritoneal cancer index; fPCI = fluorescence PCI; F = female; M = male.

	Sex	Age (y)	ASA score	TNM primary tumour	Site of primary tumour	Differentiation of primary tumour	Presentation of PC	Mean clinical PCI*	Mean fPCI**
Patient 1	F	67	2	T4N0M0	Ascending colon	Poor	Metachronous	3	1.5
Patient 2	M	68	2	T4N1M0	Caecum, sigmoid†	Well	Metachronous	7.5	2.5
Patient 3	M	26	1	T4N2M1	Sigmoid	Poor‡	Synchronous	18	14
Patient 4	M	70	2	T3N1M0	Sigmoid	Poor	Metachronous	14.5	12.5
Patient 5	M	61	2	T3N0M1¶	Rectum	Well	Metachronous	5	3.5
Patient 6	F	65	3	T4N1M1	Ascending colon	Moderate	Synchronous	8.5	6
Patient 7	F	49	2	T3N0M1	Sigmoid	Moderate	Synchronous	4.5	1

Clinical PCI and fPCI was obtained in all seven patients (Table 1). Median clinical PCI was 7.5 (range 3–18) and the median fPCI was 3.5 (1–14). The mean PCI was lower in all patients when using molecular fluorescence imaging, compared with by normal clinical means by 4 points (Supplemental table 1).

Figure 1 shows representative images of fluorescence signals detected intraoperatively. In two patients, additional tumour tissue was detected by molecular fluorescence-guided surgery that was initially missed by the surgeons. In one patient, a resection surface was visualised by molecular fluorescence but missed by conventional visual inspection and palpation alone; in another patient a para-aortal lymph node was similarly missed by conventional visual inspection and palpation, but visualised by molecular fluorescence; both regions were postoperatively confirmed to be tumour positive by histopathology.

During back-table fluorescence imaging, we marked and analysed a total of 80 areas. 29 of these 80 areas were non-fluorescent while 51 were fluorescent. All of these 29 non-fluorescent areas were histologically proven tumour-negative, while 27 (53%) of the 51 fluorescent areas were found to be adenocarcinoma.

We requested a total of 79 FFPE blocks containing benign but clinically suspicious (n=53) and malignant peritoneal lesions (n=26) from the pathology department. Semi-quantitative analyses showed a median MFI in peritoneal metastases of 6338 (range 1298–13718), whereas benign lesions showed a median MFI of 748 (range 425–4728; Figure 2). Comparison of the MFI of peritoneal metastases to tumour-negative lesions revealed a tumour-to-normal ratio of 6.92 (SD 2.47). On the basis of the MFI lower limit of the peritoneal metastases (i.e., MFI of 1298), eight false-positive lesions were identified *ex vivo*, of which four could be attributed to the presence of foreign inclusion bodies such as suturing material from previous surgery. The other remaining false-positive lesions were fibrotic or highly vascular reactive lesions.

Tumour areas co-localised completely with fluorescence signals and VEGF expression in all peritoneal metastases. Furthermore, no fluorescence could be seen in the peritoneal metastases obtained from previous laparoscopy (n=4) without tracer injection and in most of the clinically suspicious but benign lesions (Figure 2). VEGF expression was seen in all peritoneal metastases, with a median H score of 257.5 (IQR 200–285). Eight (31%) of the 26 peritoneal lesions showed intermediate VEGF expression, whereas the remaining 18 (69%) showed high VEGF expression (Supplemental figure 2). Representative examples of the *ex-vivo* histopathological analyses are depicted in the supplemental material (Supplemental figure 3-5).

Figure 1. Intraoperative imaging by white light imaging, molecular fluorescence-guided surgery, and overlay images. Intraoperative detection of a peritoneal metastasis detected by visual inspection and palpation (A; arrow) and fluorescence-guided surgery (B and C; arrow). In the same patient, fluorescence detection of a tumour-positive resection surface of a recurrent sigmoid carcinoma (E, F; arrow) that was initially missed by visual inspection and palpation alone (D); the fluorescent dye excreted by the kidney within the ureter (triangle) is also depicted. (G–I) After resection of a para-aortal lymph node, three small fluorescent spots were detected in adjacent metastatic lymph nodes. (J–L) A clinically suspicious but non-fluorescent benign lesion (arrow), which was confirmed by histopathology. A video of the procedure is available online (see web video).

Figure 2. Mean fluorescent intensity of tissue slides. The cut-off mean fluorescent intensity value for the tumour versus non-tumour was set at 1298 (dotted line). Lesions indicated as false positives ($n=8$) were due to foreign body material such as previous suturing material or highly fibrotic and vascularised lesions. Patient samples of peritoneal metastases taken before tracer injection were included as control specimen for tumour ($n=4$) and non-tumour lesions ($n=3$).

DISCUSSION

In patients with peritoneal carcinomatosis of colorectal origin who are eligible for cytoreductive surgery and HIPEC with curative intent, intraoperative assessment of the tumour load is important for two reasons: to carefully select patients before a potentially debilitating surgical procedure; and to achieve a complete cytoreduction, which is associated with increased overall survival.⁵ In the present study, we showed for the first time that molecular fluorescence-guided surgery using bevacizumab-IRDye800CW is safe and feasible, and we showed, on a macroscopic and microscopic level, that bevacizumab-IRDye800CW accumulates in peritoneal metastases. Moreover, we found that all non-fluorescent lesions proved to be benign. Consequently, the added value of our imaging technique might be that if a surgeon identifies a suspicious peritoneal lesion by visual inspection and palpation that is non-fluorescent, it could safely be left in situ. Therefore, molecular fluorescence-guided surgery could potentially prevent overtreatment in cytoreductive surgery and HIPEC for the treatment of peritoneal carcinomatosis of colorectal origin.

At present, tumour load is estimated by the PCI on the basis of visual inspection and palpation alone. To our knowledge, no data exist on the diagnostic accuracy of the clinical PCI. Nevertheless, a PCI of 20 is deemed to be the cut-off value for the selection of patients that potentially could be cured by cytoreductive surgery and HIPEC. We expected to see a higher fPCI than we found because we reasoned more lesions would be detected with molecular fluorescence-guided surgery than by conventional means. Counterintuitively, despite finding new fluorescent lesions, the fPCI was lower than the clinical PCI in all seven patients because more clinically suspicious lesions were non-fluorescent, and so could be interpreted as benign. Patients with a PCI higher than 20 might therefore be

falsely rejected for cytoreductive surgery and HIPEC in present clinical practice. Moreover, application of molecular fluorescence-guided surgery in a fluorescence laparoscopic setting might enable improved patient selection and thus avoid unnecessary laparotomy and potential complications. However, present fluorescence laparoscopy systems have to show sufficient sensitivity to detect our targeted tracer after microdosing.

In cancer surgery it is crucial to leave no residual disease. However, resection of multiple different organs is associated with substantial morbidity.¹⁵ In this study, back-table imaging of the fresh surgical specimen confirmed that all non-fluorescent areas were indeed benign. As such, no false-negative lesions were detected. Consequently, if a suspicious peritoneal lesion detected by visual inspection and palpation is non-fluorescent, the surgeon can be reasonably confident that this lesion is benign and can be safely left in situ. For example, in one patient, we detected intraoperatively clinically suspicious but non-fluorescent lesions on the small bowel. Conforming to present clinical practice, we did a partial small bowel resection. However, these non-fluorescent lesions proved to be benign after pathological examination (for an example of a benign lesion see Supplemental figure 3 and Supplemental video 1). This finding is an example of an unnecessary resection of benign tissue that potentially will be prevented by molecular fluorescence-guided surgery in the future. Moreover, the detection of a tumour-positive resection surface and para-aortal lymph node by fluorescence imaging suggests that this imaging technique is able to find malignant tissue that was initially missed by two experienced HIPEC surgeons. In the future, this intraoperative feedback could potentially lead to the direct application of intraoperative therapeutic measures, such as intraoperative brachytherapy in case of a positive resection surface.

We recorded a false-positive rate of 47% during back-table fluorescence imaging. The high false-positive rate might be explained by technical limitations of the intraoperative camera system in distinguishing true positive signals from signals caused by, for example, foreign body material or scarring. The fluorescence camera system of our setup is still in development and improvements with regard to interpretation of fluorescence signals and intraoperative quantification have not been finalised yet. Improvements such as multispectral subtraction techniques might be useful in separating the fluorescence spectrum of our near-infrared dye from other fluorescence signals, such as foreign body inclusions. Furthermore, by improving intraoperative quantification of fluorescence signals, a threshold could be set to distinguish fluorescence intensities derived from benign and tumour lesions. An increased tracer dose has potential to help differentiate true-positive from false-positive fluorescence signals. At present, three clinical dose-finding studies are ongoing, with tracer doses up to 50 mg in patients with familial adenomatous polyps (NCT02113202), breast cancer (NCT02583568), and pancreatic cancer (NCT02743975). These results will be taken into consideration when designing a subsequent phase 2 study.

The present phase 1 feasibility study was underpowered to reliably determine the diagnostic accuracy of our imaging technique, even if all ten patients according to the study protocol had been included (Supplemental Material, p1). Although the small number of patients restricts definitive conclusions, the high number of analysed specimens

provided sufficient data to confirm the potential of molecular fluorescence-guided surgery during cytoreduction and HIPEC. A sufficiently powered multicentre, phase 2 study is being designed under the auspices of the Dutch Peritoneal Oncology Group to determine the diagnostic accuracy in terms of sensitivity and specificity of molecular fluorescence-guided surgery using bevacizumab-IRDye800CW during laparotomy and thus separately in a near-infrared fluorescence laparoscopic setting.

In conclusion, our study showed that molecular fluorescence-guided surgery in peritoneal carcinomatosis of colorectal origin using bevacizumab-IRDye800CW is technically feasible, safe, and has the potential to improve patient selection and cytoreduction during cytoreductive surgery and HIPEC. Although molecular fluorescence-guided surgery was associated with more false-positive results than initially expected, it is promising to see that in the absence of fluorescence no residual disease was found. These results have led to the design of a subsequent national, phase 2, diagnostic accuracy study to provide supportive data for changing the standard of care in patients with peritoneal carcinomatosis of colorectal cancer origin undergoing cytoreduction and HIPEC using molecular fluorescence-guided surgery.

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DECLARATION OF INTEREST

MK reports grants from the FP-7 Framework Programme BetaCure grant no. 602812, during the study. WBN reports grants from SurgVision BV during the study. VN is a co-founder and equity holder of SurgVision BV. GMvD reports grants from SurgVision BV and the EU FP7 Research programme (no. 602812) BetaCure, during the study; and is a member of the scientific advisory board of SurgVision BV. All other authors declare no competing interests.

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REFERENCES

- 1 Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *Ca Cancer J Clin* 2015; 65: 87–108.
- 2 Koppe MJ, Boerman OC, Oyen WJ, Bleichrodt RP. Peritoneal Carcinomatosis of Colorectal Origin. *Ann Surg* 2006; 243: 212–22.
- 3 Verwaal VJ, Bruin S, Boot H, van Slooten G, van Tinteren H. 8-Year Follow-up of Randomized Trial: Cytoreduction and Hyperthermic Intraperitoneal Chemotherapy Versus Systemic Chemotherapy in Patients with Peritoneal Carcinomatosis of Colorectal Cancer. *Ann Surg Oncol* 2008; 15: 2426–32.
- 4 Ihemelandu CU, McQuellon R, Shen P, Stewart JH, Votanopoulos K, Levine EA. Predicting Postoperative Morbidity Following Cytoreductive Surgery with Hyperthermic Intraperitoneal Chemotherapy (CS+HIPEC) with Preoperative FACT-C (Functional Assessment of Cancer Therapy) and Patient-Rated Performance Status. *Ann Surg Oncol* 2013; 20: 3519–26.
- 5 Sugarbaker PH, Ryan DP. Cytoreductive surgery plus hyperthermic perioperative chemotherapy to treat peritoneal metastases from colorectal cancer: standard of care or an experimental approach? *Lancet Oncol* 2012; 13: e362–9.
- 6 Klumpp B, Schwenzer N, Aschoff P, et al. Preoperative assessment of peritoneal carcinomatosis: intraindividual comparison of 18F-FDG PET/CT and MRI. *Abdom Imaging* 2013; 38: 64–71.
- 7 van Dam GM, Themelis G, Crane LM, et al. Intraoperative tumor-specific fluorescence imaging in ovarian cancer by folate receptor- α targeting: first in-human results. *Nat Med* 2011; 17: 1315.
- 8 de Boer E, Harlaar N, Taruttis A, et al. Optical innovations in surgery. *Brit J Surg* 2015; 102: e56–72.
- 9 Weissleder R, Ntziachristos V. Shedding light onto live molecular targets. *Nat Med* 2003; 9: 123–8.
- 10 van Oosten M, Crane L, Bart J, van Leeuwen FW, van Dam GM. Selecting Potential Targetable Biomarkers for Imaging Purposes in Colorectal Cancer Using TARGET Selection Criteria (TASC): A Novel Target Identification Tool. *Transl Oncol* 2011; 4: 71–82.
- 11 de Cuba E, de Hingh I, Sluiter N, et al. Angiogenesis-Related Markers and Prognosis After Cytoreductive Surgery and Hyperthermic Intraperitoneal Chemotherapy for Metastatic Colorectal Cancer. *Ann Surg Oncol* 2016; 23: 1601–8.
- 12 ter Weele EJ, van Scheltinga A, Linssen MD, et al. Development, preclinical safety, formulation, and stability of clinical grade bevacizumab-800CW, a new near infrared fluorescent imaging agent for first in human use. *Eur J Pharm Biopharm* 2016; 104: 226–34.
- 13 Themelis G, Yoo J, Soh K-S, Schulz R, Ntziachristos V. Real-time intraoperative fluorescence imaging system using light-absorption correction. *J Biomed Opt* 2009; 14: 064012–064012–9.
- 14 Sugarbaker PH. Peritonectomy Procedures. *Ann Surg* 1995; 221: 29–42.
- 15 Kuijpers AM, Aalbers AG, Nienhuijs SW, et al. Implementation of a Standardized HIPEC Protocol Improves Outcome for Peritoneal Malignancy. *World J Surg* 2015; 39: 453–60.

SUPPLEMENTAL MATERIAL

Motivation for deviation of study protocol

According to the study protocol the primary objectives of this phase I feasibility study were to determine the safety and feasibility in terms of diagnostic accuracy (i.e. sensitivity and specificity) for the intraoperative detection of peritoneal carcinomatosis of colorectal origin by fluorescence imaging. As the protocol states, we intended to include ten patients in this study and to collect a minimum of ten samples per patient during back table imaging of the fresh surgical specimen. However, the enrolment of patients was stopped after seven patients for multiple reasons as described below.

Unfortunately, one of our study patients died unexpectedly of an asystole, most probably based on a cardiovascular thromboembolic event. Therefore, the study was put on hold to perform in-depth safety analyses by the DSMB and IRB of the UMCG. During the four months the study was put on hold we finalized all of the *ex vivo* analyses regarding the aims of this study. We concluded that this phase I feasibility study was underpowered to reliably determine the diagnostic accuracy of our imaging technique. Retrospectively, we underestimated the fact that we would also include patients with a lower tumour load. Therefore, it would not have been possible to collect sufficient data per patient to determine a statistically significant diagnostic accuracy, even if we would have included 10 patients. Furthermore, during the four months the study was put on hold, a critical change was made in the tracer production process at the pharmacy department, resulting in a second-generation tracer with different optical properties. The modification of the tracer might have influenced imaging results, causing difficulties in data comparison. Moreover, the same tracer that was used in the first seven patients was not available anymore, which was another argument to stop the inclusion after seven patients.

A sufficiently powered national multicentre phase II diagnostic accuracy study is being initiated under the auspices of the Dutch Peritoneal Oncology Group (DPOG), to determine the diagnostic accuracy in terms of sensitivity and specificity of MFGS using bevacizumab-IRDye800CW.

Supplemental figures

Supplemental figure 1. The concept of molecular fluorescence guided surgery (MFGS). A near-infrared fluorophore (IRDye800CW) is conjugated to an antibody (bevacizumab) targeting VEGF-A (panel A). Two days prior to surgery, 4.5 mg of the GMP produced fluorescent targeted tracer was injected intravenously (panel B). During cytoreductive surgery, a molecular fluorescence camera system is used to provide the surgeon with real-time feedback on possible fluorescence signals via a monitor (panel C).

Supplemental figure 2. Anti-VEGF immunohistochemistry of peritoneal metastases. The median H-value of the VEGF IHC scoring of peritoneal metastases is 257.5 (interquartile range 200 – 285; panel a). 31% of the peritoneal metastases showed intermediate VEGF expression, whereas 69% showed high VEGF expression (panel b). Panel c demonstrates a representative example of a peritoneal metastasis with high VEGF expression.

Supplemental figure 3. Co-localization of white-light imaging (colour FFPE-block), fluorescence flatbed scanning of FFPE-block (NIRF FFPE-block) and 10 µm slide (NIRF 10 µm), Hematoxylin and Eosin staining (H/E 10 µm) and VEGF immunohistochemistry (VEGF 4 µm). Tumour areas co-localized completely with fluorescence signals and VEGF expression in peritoneal metastases (panels a-e). No fluorescence signals nor VEGF expression could be observed in a clinically suspicious but benign lesion (panels f-j). No fluorescence could be observed in the peritoneal metastasis obtained from previous laparoscopy without tracer injection (panels k-o). Tumour tissue is delineated by yellow and black lines. FFPE = Formalin-fixed, paraffin-embedded, NIRF = Near Infrared Fluorescence.

Supplemental figure 4. Mesoscopic imaging and co-localization of the bevacizumab-IRDye800CW tracer in peritoneal carcinomatosis of colorectal origin and H/E staining for tumour segmentation. Conventional white light colour imaging of a FFPE tissue block (panel a), H/E staining of a 10 µm slide of the same PPFE block (panel b), and a 4x magnification of the same slide (panel c). Near-infrared fluorescence (NIRF) imaging of the FFPE tissue block (panel a,d), subsequent 10 µm slide (panel e) and 4x magnification (panel f). The tumour lesion is located in the sub serosa and is segmented by a yellow line as based on standard H/E histopathology. NIRF signals co-localize with the tumour and its margin, but not with the benign microvilli of the bowel wall. FFPE = formalin-fixed, paraffin embedded. NIRF = near infrared fluorescence, H/E = hematoxylin and eosin staining, * = tumour tissue, white triangle = normal villi.

Supplemental figure 5. Fluorescence microscopy of bevacizumab-IRDye800CW in a peritoneal metastasis of colorectal origin. Near-infrared fluorescence microscopy image of a 4 µm tissue slide (panel a: blue = Hoechst nucleic acid stain, red = NIR fluorescence channel, bevacizumab-IRDye800CW; white arrow indicates tumour cells) and corresponding hematoxylin/eosin stained image (panel b, black arrow indicates tumour cells).

Supplemental table 1. Inter-observer variation of the (fluorescence) Peritoneal Cancer Index. *PCI* = Peritoneal cancer index; *fPCI* = fluorescence *PCI*.

	Surgeon 1		Surgeon 2		Mean <i>PCI</i>	Mean <i>fPCI</i>
	<i>PCI</i>	<i>fPCI</i>	<i>PCI</i>	<i>fPCI</i>		
Patient 1	2	1	4	2	3	1.5
Patient 2	8	3	7	2	7.5	2.5
Patient 3	20	14	16	14	18	14
Patient 4	16	13	13	12	14.5	12.5
Patient 5	6	5	4	2	5	3.5
Patient 6	8	6	9	6	8.5	6
Patient 7	4	1	5	1	4.5	1

Supplemental video

Supplemental video 1. Molecular fluorescence-guided surgery of a non-fluorescent benign lesion, back-table fluorescence imaging of a fluorescent metastasis, and molecular fluorescence-guided surgery of a false-positive fluorescent lesion

Supplemental protocols

A - Immunohistochemical staining protocol for VEGF (paraffin slides)

The following steps were used for VEGF-A staining. First the slides were deparaffinised using xylene (2x5 minutes) and air dried. After NIRF flatbed scanning, endogen peroxidase blocking was performed using (4.5 ml 30% H2O2 in 45.5 ml PBS) for 30 minutes followed by incubation with polyclonal rabbit anti-human VEGF-A (RB9031, ThermoScientific) (1:300). This was followed by poly-HRP-anti Ms/Rb/Rt IgG (BrightVision) staining (2x30 minutes) at room temperature and the peroxidase reaction using diaminobenzidine for 10 minutes. Between all the steps PBS was used for washing the slides except after the peroxidase reaction, when distilled water was used (3x5 minutes). A counterstaining was performed using Mayer's hematoxylin followed by dehydration with alcohol (70%-96%-100%). After air-drying (30 minutes), the slides were embedded using mounting media.

B - VEGF scoring

Peritoneal metastases were independently scored by two observers (MK and SJJ). Tissue slides of all peritoneal metastases were scored separately for staining intensity on a scale ranging from 0 – 3 (absent – strong VEGF staining) and the percentage of cells stained. Next, H-scores were calculated on a continuous scale ranging from 0 – 300 using the following formula: 1x (percentage of cells weakly stained [1+]) + 2x (percentage of cells moderately scored [2+]) + 3x (percentage of cells strongly stained [3+]). H-score categories were defined as follows: 0-100 = negative/low; 101-200 = intermediate; 201-300 = high.

C – Hoechst staining protocol (nucleic acid stain)

The following steps were used for Hoechst staining. First the slides were deparaffinised using xylene (2x5 minutes) and subsequently air dried. A Hoechst solution (100 µl of 0.5 µg/ml, stored at 4 °C; 33258, Invitrogen) was pipetted on the deparaffinised slide for 15 minutes followed by three washing steps, first using (4.5 ml 30% H2O2 in 45.5 ml PBS) followed by distilled water. The stained slides were air dried in the dark and mounted under a cover-glass using modified Kaiser's glycerine (preheated in a 60 °C incubator) and dried overnight.

Fluorescence-guided imaging for resection margin evaluation in head and neck cancer patients using cetuximab-800CW: A quantitative dose-escalation study

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ABSTRACT

Rationale

Tumor-positive resection margins are present in up to 23% of head and neck cancer (HNC) surgeries, as intraoperative techniques for *real-time* evaluation of the resection margins are lacking. In this study, we investigated the safety and potential clinical value of fluorescence-guided imaging (FGI) for resection margin evaluation in HNC patients. We determined the optimal cetuximab-800CW dose by quantification of intrinsic fluorescence values using multi-diameter single-fiber reflectance, single-fiber fluorescence (MDSFR/SFF) spectroscopy.

Methods

Five cohorts of three HNC patients received cetuximab-800CW systemically: three single dose cohorts (10, 25, 50 mg) and two cohorts pre-dosed with 75 mg unlabeled cetuximab (15 or 25 mg). Fluorescence visualization and MDSFR/SFF spectroscopy quantification were performed and correlated to histopathology.

Results

There were no study-related adverse events higher than Common Terminology Criteria for Adverse Events grade-II. Quantification of intrinsic fluorescence values showed a dose-dependent increase in background fluorescence in the single dose cohorts ($p < 0.001$, $p < 0.001$), which remained consistently low in the pre-dosed cohorts ($p = 0.6808$). Resection margin status was evaluated with a sensitivity of 100% (4/4 tumor-positive margins) and specificity of 91% (10/11 tumor-negative margins).

Conclusion

A pre-dose of 75 mg unlabeled cetuximab followed by 15 mg cetuximab-800CW was considered the optimal dose based on safety, fluorescence visualization and quantification of intrinsic fluorescence values. We were able to use a lower dose cetuximab-800CW than previously described, while remaining a high sensitivity for tumor detection due to application of equipment optimized for IRDye800CW detection, which was validated by quantification of intrinsic fluorescence values.

INTRODUCTION

Head and neck cancer (HNC) is the sixth most common cancer worldwide, with an incidence of over 550,000 and around 300,000 cancer-related deaths each year.¹ The most common histological subtype is head and neck squamous cell cancer (HNSCC), accounting for more than 90% of all HNCs. Depending on anatomical location, the primary treatment of HNSCC consists of surgical resection or (chemo)radiotherapy.²⁻⁷ In case of surgical treatment, tumor-negative resection margins are essential for local control and consequently improve survival. A tumor-positive resection margin in head and neck oncology is generally defined as viable tumor cells within 1mm of the resection margin. Tumor-negative resection margins reduce the need for adjuvant treatment, while a tumor-positive resection margin often involves treatment with cisplatin-based chemo-radiation, which severely increases the risk of sequelae.

Despite improving pre- and intraoperative imaging techniques, no accurate clinically approved tools are available to discriminate between tumor and surrounding non-tumor tissue for *real-time* margin assessment during surgery. Consequently, surgeons rely on their experience and visual and tactile information alone. Due to the intrinsic limitations of the provided information, this contributes to a tumor-positive resection margin rate that is reported to be as high as 23% in HNSCC.⁸⁻¹⁰

With the development of clinical grade small peptides and antibodies conjugated to fluorescent dyes, the field of fluorescence-guided imaging (FGI) has rapidly evolved and expanded to application in several solid tumor types.¹¹⁻¹⁴ FGI has the potential to provide *real-time* feedback on increased receptor expression in cancerous tissue and highlighting these cancer cells by binding of fluorescent labelled tracers, making it an interesting technique for intraoperative margin evaluation. Since the first clinical study on FGI in 2011, in which a conjugate of folate-FITC was used to visualize peritoneal metastasis of ovarian cancer, several studies in different tumor types and organs have been performed that further demonstrate the potential of FGI for discrimination between tumor and healthy tissue.^{13,15-19}

A promising target for targeted-FGI in HNSCC patients is the epidermal growth factor receptor (EGFR). Overexpression of EGFR in HNSCC is present in up to 90% of the cases and is associated with higher local recurrence and poorer survival rates.^{20,21} Two early phase clinical trials on FGI in HNSCC patients have already been performed, investigating a monoclonal antibody (cetuximab and panitumumab respectively) targeting EGFR, which was conjugated to the near-infrared (NIR) fluorophore IRDye800CW.^{15,22}

Since external factors such as ambient light, angle of imaging and distance from tissue to imaging device all may influence imaging results severely^{23,24}, it is hypothesized that evaluation of the excised specimen might be interesting, with encouraging results reported previously using panitumumab-800CW.^{25,26} However, although tissue absorption and scattering are minimized in the NIR spectrum (700-900 nm), these phenomena affect fluorescence imaging resulting in higher signals in highly scattering tissue or lower signals in areas that contain high concentrations of blood or in crusts of necrotic tissue for example.

To date, correction for these factors is lacking for FGI in HNC surgery, which might cause different interpretation of fluorescence results within and between patients and centers. In order to tackle the aforementioned technical issues, we applied multi-diameter single-fiber reflectance and single-fiber fluorescence (MDSFR/SFF) spectroscopy to correct for tissue optical properties in order to determine the quantitative intrinsic fluorescence values.^{23,27-30} The design of our study was adapted based on earlier reports that a pre-dose of unlabeled cetuximab improved cetuximab-800CW FGI results, most likely due to increased off-target receptor occupancy and tissue sequestration.³¹ However, to date administration of a single flat-dose of cetuximab-800CW has not been compared to administration of cetuximab-800CW preceded by an unlabeled dose of cetuximab, nor has quantification of intrinsic fluorescence values been included in dose-escalating studies to find the optimal cetuximab-800CW dose for FGI.

In this study, we determined the safety and optimal dosing regimen of cetuximab-800CW for FGI through *real-time* intraoperative fluorescence visualization and quantification of intrinsic fluorescence values and evaluated the clinical added value of back-table FGI for resection margin assessment.

METHODS

Clinical Trial design

This phase-I safety, feasibility and dose-escalation study was performed at the department of Oral & Maxillofacial Surgery of the University Medical Center Groningen (UMCG, Groningen, The Netherlands). Fifteen patients with histopathological proven HNSCC that were scheduled for tumor resection were included. Patients were identified after the HNC multidisciplinary tumor board agreed on surgery as the primary treatment modality. Written and signed informed consent was obtained prior to any study-related procedure. The study was approved by the Institutional Review Board (IRB) of the UMCG (METc 2016/395) and conducted according to the Dutch Act on Medical Research involving Human Subjects (WMO) and the principles of the Declaration of Helsinki (adapted version Fortaleza, Brazil, 2013). The trial was registered at www.clinicaltrials.gov (NCT03134846).

We performed an adapted phase-I dose-escalation study (5x3 design) adhering to the FDA guidelines (Guidance for Industry, Developing Medical Imaging Drug and Biological Products, Part 2 Clinical Indications). All fifteen patients received cetuximab-800CW four days prior to surgery. The dose-escalation study was divided into two parts. Part I involved a single intravenous (IV) administration of cetuximab-800CW (10, 25 or 50 mg single-dose). After an interim analysis, part II commenced, in which patients received 75mg unlabeled cetuximab IV 1h prior to IV administration of cetuximab-800CW (15 or 25 mg). This 1 h interval was based on previous studies on pre-dosing with cetuximab.^{15,31} The study workflow is depicted in Figure 1.

Figure 1. Study workflow. Five cohorts of three HNC patients received cetuximab-800CW systemically: three single dose cohorts (10, 25, 50 mg) and two cohorts pre-dosed with 75 mg unlabeled cetuximab (15 or 25 mg). Fluorescence visualization was performed before and after excision of the tumor in vivo. Subsequently, back-table fluorescence-guided imaging of the fresh surgical specimen was performed to evaluate the resection margin status. Visualization and quantification of fluorescence was performed during all subsequent steps of standard histopathological processing and correlated to histopathology. Abbreviations: FFPE: Formalin-Fixed Paraffin Embedded, H/E: Hematoxylin and Eosin. *75 mg unlabeled cetuximab is administered one hour prior to cetuximab-800CW administration.

GMP synthesis of cetuximab-800CW

Clinical grade cetuximab-800CW (peak excitation and emission wavelength of 778 and 795 nm respectively) was produced in the Good Manufacturing Practice (GMP) facility of the UMCG and released by a certified Qualified Person (QP). A detailed description of the production process has been described previously.³² Briefly, commercially available cetuximab (Erbix®) 5 mg/mL was conjugated to the near-infrared fluorescence dye IRDye800CW (LI-COR Biosciences, Lincoln, NE, USA) and purified using PD-10 buffer exchange columns (GE Healthcare, Chicago, IL, USA). Cetuximab-800CW was formulated in a sodium-phosphate buffer at a concentration of 1 mg/mL. All study patients received cetuximab-800CW through a single IV bolus injection.

Safety measurements

Patients underwent a medical screening procedure before enrollment in the study, consisting of measurements of vital signs, a 12-lead electrocardiogram (ECG) and laboratory tests (including a serum pregnancy test for woman of childbearing potential). Once enrolled, an ECG was performed 1 h after administration of cetuximab-800CW. Vital signs were measured before and after tracer administration (directly and after 1 h). Patients were asked for signs and symptoms before and after tracer administration (directly and after 1 h). After surgery, standard postoperative (outpatient clinic) visits were performed within two weeks. Adverse event assessment was performed according to the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) version 4.0. Serious adverse events (SAE), if present, were reported to the IRB, the independent Data Safety

Monitoring Board (DSMB) and the Dutch central committee on research involving human subjects (CCMO). SAE's were followed up until fully resolved or a stable medical situation was achieved.

Surgical procedure (standard of care)

All patients underwent tumor resection according to standard surgery protocols. Depending on preoperative TNM staging, a sentinel lymph node biopsy (lymph node mapping using the peritumoral administration of Technetium-99m-labelled nanocolloid one day prior to surgery and subsequent intraoperative detection using a gamma-probe) or a lymph node dissection was performed. Based on our previous experience in the UMCG with application of FGI, there was minimal interference with standard of care.^{13,16,18}

***In vivo* fluorescence imaging**

Surgical procedures were performed according to standard of care and were prioritized over any study-related procedures. Visualization of fluorescence was performed prior to incision of the primary tumor and directly after excision of the surgical wound bed. During fluorescence imaging, the ambient light in the surgical theatre was dimmed to minimize influence on fluorescence imaging results. The use of methylene blue in sentinel node procedures was avoided. The use of fluorescent skin markers and (green) fluorescent sterile drapes was minimized to prevent interference with fluorescence imaging results.¹⁸ It should be noted that *in vivo* imaging of the tumor in the oral cavity is only performed to visualize fluorescence and observe fluorescence patterns. All measurements of fluorescence intensities are performed on the excised specimen.

Fluorescence cameras

Two intraoperative fluorescence imaging devices were used in this study to detect cetuximab-800CW. Depending on the location of the tumor, either a custom build fluorescence endoscopy platform (SurgVision BV., Groningen, The Netherlands) attached to a flexible nasendoscope (Karl Storz, Tuttlingen, Germany), or an intraoperative fluorescence camera system (Explorer Air®, SurgVision BV., Groningen, The Netherlands) was used during surgery. Both systems provide *real-time* simultaneous fluorescence and white light (color) images and videos. Fluorescence is excited by either a NIR laser (fluorescence nasendoscopic system) or light emitting diodes (Explorer Air®), both of which have an excitation peak specific for cetuximab-800CW detection. Filtered white light is used to provide illumination for the color images. A software user interface is provided to allow the user control over camera settings and to display the color and fluorescence images. As output, both snapshots as well as videos can be recorded and stored in TIFF format. The working distance of the imaging system above the surgical field was set at 3-5 cm for the fluorescence nasendoscopic system and 20 cm for the Explorer Air®. All images were initially obtained with a fluorescence exposure time of 50 ms and a set gain of 300, which could be adjusted depending on fluorescence imaging results.

Back-table FGI and specimen processing

Immediately after excision, the surgical specimen was marked using sutures for orientation and cross-correlation with histopathology. Subsequently, back-table FGI was performed of all resection planes of the fresh surgical specimen. Two back-table FGI devices were used in parallel, the Explorer Air® coupled to a closed-field imaging box (Vault®, SurgVision BV., Groningen, The Netherlands) and a PEARL-trilogy® imager with a larger adapted sample stage (LI-COR BioSciences Inc., Lincoln, NE, USA). The Pearl-trilogy® detects fluorescence using a CCD camera in the NIR wavelength (peak excitation 785 nm, peak emission 820 nm). The field of view of 11.2 cm x 8.4 cm and the focus point can be adjusted based on specimen height. Fluorescence was scored at the resection margin by physicians blinded for histology results and scored positive if the TBR was higher than two (the specimen was used as its own internal control).

After back-table FGI, the specimen was formalin fixed for at least 24 h, depending on specimen size, after which the specimen was inked to mark resection margins on final histopathology with black and blue ink.

The formalin-fixed surgical specimen was serially sliced into approximately 0.4 cm thick tissue slices. White-light images were acquired during and directly after tissue slicing for orientation purposes. After slicing, fluorescence imaging was performed on both sides of each tissue slice using the PEARL-trilogy®. Subsequently, a pathologist blinded for the fluorescence images selected regions of interest based on gross examination by visual inspection and palpation, which were further embedded in paraffin blocks for standard histopathological analysis. In case of the presence of bone, tissue was decalcified before further processing. A standardized workflow was used in order to cross-correlate final histopathology results with recorded fluorescence images of tissue slices of interest.¹⁸ Subsequently, hematoxylin and eosin (H/E) staining was performed on 4 µm tissue sections for histopathological analysis and analyzed by a board-certified pathologist blinded for fluorescence imaging results. A tumor-positive resection margin was defined as viable tumor tissue within 1 mm of the resection margin according to European guidelines in HNSCC treatment.³³

Ex vivo MSDFR/SFF spectroscopy

The MSDFR/SFF spectroscopy device acquires two reflectance spectra by using two different optical fibers and subsequently one raw fluorescence spectrum, as described previously.¹⁸ Briefly, the scattering and absorption coefficients are determined from the two reflectance spectra. These are then used to calculate the intrinsic fluorescence values ($Q \cdot \mu_{a,x}^f$) of cetuximab-800CW, by correcting the raw fluorescence spectrum for the calculated tissue optical properties.³⁴ Intrinsic fluorescence values ($Q \cdot \mu_{a,x}^f$) of cetuximab-800CW are defined as a product of the quantum efficiency across the emission spectrum, $Q[-]$, where Q is the fluorescence quantum yield of IRDye-800CW and $[mm^{-1}]$ is the tracer absorption coefficient at the excitation wavelength. The data is collected using direct contact measurements. All measurements were repeated in triplicate and median values were calculated per measurement location.

We calculated the concentration of IRDye800CW based on an assumed extinction coefficient (31120.4 mm^{-1}), fluorescence quantum yield (0.09) and the cetuximab-800CW load factor (1:2) for each cohort¹² *ex vivo* in the tissue specimens.

Ex vivo validation of fluorescence intensities and localization

PEARL-trilogy® images of tissue slices that contained tumor tissue and non-tumor tissue were used to calculate the mean fluorescence intensity (MFI) of both tumor and non-tumor tissue.

A board-certified pathologist, blinded from the fluorescence images, drew region of interests (ROI) of areas that contained tumor on $4 \mu\text{m}$ H/E slides and subsequently overlaid on the corresponding tissue slice. MFIs were calculated based on these ROIs on the tissue slices of both tumor tissue and non-tumor tissue (i.e. all tissue in the tissue slice diagnosed as non-tumor tissue). A tumor-to-background-ratio (TBR) was calculated by dividing tumor ROI (MFI tumor) with the background ROI (MFI non-tumor tissue) per tissue slice. Median TBR values were calculated on a per patient base.

Immunohistochemical analysis was performed on $4 \mu\text{m}$ thick tissue sections which contained tumor and non-tumor tissue to correlate fluorescence intensities with EGFR expression (VENTANA Benchmark Ultra anti-EGFR (Clone 3C6), Roche, Basel, Switzerland). The EGFR-membrane expression was evaluated following a pre-defined scoring system (0, +, ++ or +++) by a board-certified pathologist.

An inverted microscope (DMI6000B, Leica Biosystems GmbH, Wetzlar, Germany) was used for fluorescence microscopy with a pixel size of $6.45 \mu\text{m}$ and a field of view of $120 \times 120 \text{ mm}$. To optimize NIR visualization, the microscope was equipped with a NIR LED light source ranging up to 900 nm (X-Cite 200DC, Excelitas Technologies, Waltham, MA, USA), a NIR filter set (microscope two band-pass filters $850\text{--}890 \text{ m--}2\text{p}$ and a long-pass emission filter HQ800795LP; Chroma Technology Corp, Bellows Falls, VT, USA) and a monochrome DFC365 FX fluorescence camera (1.4 M Pixel CCD , Leica Biosystems GmbH). An acquisition time of 12 s was used for images of the 800 nm channel.

Cetuximab-800CW tracer integrity

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed both on blood samples of the included patients collected on day four and on lysate of fresh frozen tumor tissue to ensure intactness of the cetuximab-800CW conjugate, as previously described (ProSieve® Quadcolor™ Protein Markers, Lonza Rockland Inc., ME, USA and Mini-Protein TGX Precast Protein Gel, Bio-Rad Laboratories Inc., California, USA).^{18,35} Results were compared with labeled and unlabeled cetuximab. The gel was scanned with the Odyssey CLX® flatbed scanner (LI-COR Biosciences Inc. Lincoln, NE, USA) at the 800 nm channel.

Statistical analysis

Descriptive statistics were performed on the patient demographics. MFI was calculated using ImageJ Fiji (version 2.0.0-rc-68/1.52h) as total counts per ROI pixel area in both tumor and non-tumor tissue. Data was tested for Gaussian distribution using Anderson-Darling and Shapiro-Wilk test; none of the data was normally distributed. Differences between paired and unpaired data was tested using a Wilcoxon test and Mann-Whitney test respectively. Data is presented as median values with interquartile ranges. For statistical analysis and graph design, GraphPad Prism (version 8.0, GraphPad Software Inc, San Diego, California, USA) was used.

RESULTS

Fifteen patients with HNSCC were included in this study. The age of the patients ranged from 48 to 86 years. Administration of cetuximab-800CW was considered to be well tolerated in all dose-cohorts, with five possibly related adverse events throughout the study that were all limited to CTCAE grade I events (Table 1 and Table S1). The grade II event (i.e. bronchospasm) was considered not to be imaging agent related but iatrogenic since the administration speed was accidentally set 10x higher than recommended.

Table 1. Patient and surgical specimen characteristics. Patient and surgical specimen characteristics are depicted per cohort. Potential related adverse events are scored according to the Common Terminology Criteria for Adverse Events. *Tumor-negative margin: >5 mm; tumor-positive surgical margin: <1 mm. Abbreviations: CTX: Cetuximab.

	10 mg CTX-800CW N=3	25 mg CTX-800CW N=3	50 mg CTX-800CW N=3	75 mg CTX + 15 mg CTX- 800CW N=3	75 mg CTX + 25 mg CTX- 800CW N=3
Patient characteristics					
Age (mean years, range)	68 (59-73)	62 (56-71)	63 (48-86)	63 (50-78)	55 (49-64)
Males (number, percentage)	2 (53%)	3 (100%)	1 (33%)	1 (33%)	1 (33%)
Weight (mean kg, range)	79 (45-104)	77 (57-97)	78 (93-97)	97 (81-140)	82 (58-122)
Safety					
Grade I	2	2	0	1	0
Grade II	0	0	0	1	0
Tumor histology					
HNSCC	3	3	3	3	3
Size					
Max Diameter (mean $\pm$ SD mm)	34 ($\pm$ 20)	20 ($\pm$ 7.5)	18 ($\pm$ 7.0)	30 ($\pm$ 11)	26 ($\pm$ 18)
Depth of Invasion (mean $\pm$ SD mm)	11 ($\pm$ 3.6)	6 ($\pm$ 1.4)	4 ($\pm$ 0.8)	5 ($\pm$ 3.1)	6 ($\pm$ 5.1)
Surgical margin*					
Tumor-negative	2	2	2	2	3
Tumor-positive	1	1	1	1	0

Evaluation of resection margin status

Four patients were diagnosed with a tumor-positive resection margin (i.e. <1 mm) based on standard histopathology (Table 1). During back-table FGI of the fresh surgical specimens, all four tumor-positive resection margins were correctly identified (sensitivity 100%; Figure 2 and Table 2). *In vivo* visualization of the wound bed did not show remaining fluorescent lesions. This was in line with final histopathology, since all four tumor-positive resection margins were based on the presence of viable tumor cells within 0.3-1 mm of the resection margin, suggesting there was no residual tumor in the wound bed.

Figure 2. Representative example of back-table FGI of a tumor-positive resection margin. Back-table fluorescence-guided imaging (FGI) of a fresh surgical specimen, with a clearly localized increased fluorescent lesion, indicating a tumor-positive ventral resection margin of a tumor located on the floor of the mouth upon final histopathology. The tumor-positive resection margin on the tissue slice and section (red arrows) correspond to the location on the fresh surgical specimen (yellow lines). Abbreviations: H/E: Hematoxylin and Eosin. EGFR: Epidermal Growth Factor Receptor.

Table 2. Contingency table of back-table fluorescence-guided imaging results. All four tumor-positive resection margins were correctly identified using back-table fluorescence-guided imaging (FGI) of the fresh surgical specimens. In addition, ten tumor-negative resection margins were correctly identified. One tumor-negative resection margin was identified as tumor-positive, based on back-table FGI. Abbreviations: FGI: Fluorescence- guided imaging.

	Tumor-positive resection margin	Tumor-negative resection margin	Total
Back-table FGI positive	4	1	5
Back-table FGI negative	0	10	10
Total	4	11	15

Figure 3. *In vivo* fluorescence visualization during surgery. **A:** Representative fluorescence imaging using the nasendoscopic fluorescence imaging system of a buccal HNSCC tumor. **B:** Representative fluorescence imaging using Explorer Air® fluorescence imaging system of a HNSCC tumor of the tongue. Yellow arrows indicate a separate second tumor lesion diagnosed using cetuximab-800CW fluorescence.

Eleven out of fifteen patients were diagnosed with a tumor-negative resection margin upon final histopathology (Table 1). Back-table FGI of the fresh surgical specimens showed no fluorescent lesions on the resection margins in 10/11 patients (specificity 91%; Table 2). The remaining resection margin (2 mm) was evaluated as tumor-positive based on a fluorescent lesion (Figure S1).

In addition, one satellite tumor lesion was detected during *in vivo* FGI, which was located anterior of the main tumor lesion (Figure 3B, yellow arrows), highlighting the potential added value of *real-time* intraoperative FGI. Fluorescence imaging procedures prolonged the standard procedure with approximately 10 min and did not interfere with the standard of care. Difficult and easily accessible tumors and surgical cavities could be visualized *in vivo* using the flexible nasendoscopic camera and the Explorer Air® respectively (Figure 3A-B and Video S1).

Visualization and quantification of cetuximab-800CW fluorescence

To objectivate the potential of cetuximab-800CW for the discrimination of tumor tissue versus non-tumor tissue, we first calculated the tumor fluorescence of the ROI of tissue slices (N=61). Here, significantly higher fluorescence intensities in tumor tissue compared to non-tumor tissue were detected for all cohorts (TBR 10 mg: 1.61 ± 0.93 , $p=0.0312$; 25 mg: 2.02 ± 0.55 , $p=0.0078$; 50 mg: 1.81 ± 0.32 , $p<0.0001$; 75 + 15 mg: 3.06 ± 0.43 , $p=0.0010$; 75 + 25 mg: 3.10 ± 2.53 , $p=0.0005$; Figure 4A-B and Table 3), with increasing TBRs in the pre-dosed cohorts. To further validate these findings, quantification of the intrinsic fluorescence values by MDSFR/SFF on tissue slices (N=46) confirmed these findings in all dose-cohorts (TBRs 10 mg: 2.86 ± 0.29 , $p=0.0312$; 25 mg: 1.99 ± 1.62 , $p=0.0078$; 50 mg: 1.48 ± 0.52 , $p<0.0001$; 75 + 15 mg: 2.50 ± 0.19 , $p=0.0010$; 75 + 25 mg: 2.81 ± 1.27 , $p=0.0005$; Figure 4C and Table 3).

Figure 4. Ex vivo correlation and quantification of cetuximab-800CW fluorescence. **A:** Tumor tissue is delineated on Hematoxylin and Eosin (H/E) staining, which correlates to the fluorescence imaging results on the tissue slices and EGFR expression on tissue slice containing tumor (upper row) and a tumor-positive resection margin (middle row), whereas a non-tumor tissue slices shows low fluorescence intensities and no EGFR expression (lower row). Red arrows indicate a tumor-positive margin of 0.3 mm. **B:** Tumor tissue showed significantly increased fluorescence intensities compared to non-tumor tissue on fluorescence images of tissue slices. **C:** Intrinsic fluorescence values were significantly higher in tumor tissue compared to non-tumor tissue. * 75 mg cetuximab is administered one hour prior to cetuximab-800CW administration. Dots represent median intrinsic fluorescence values per measurements location. Error bars represent median values and interquartile range. Abbreviations: H/E: Hematoxylin and Eosin. EGFR: Epidermal Growth Factor Receptor.

Table 3. *Ex vivo tumor-to-background ratio per dose cohort. Median tumor-to-background ratios shown both for fluorescence visualization using a closed-field imaging device and quantification of intrinsic fluorescence values using MDSFR/SFF spectroscopy.*

Dose-cohort	TBR	
	Visualization	Spectroscopy
10mg	1.61 ± 0.93	2.86 ± 0.29
25mg	2.02 ± 0.55	1.99 ± 1.62
50mg	1.81 ± 0.32	1.48 ± 0.52
15mg*	3.06 ± 0.43	2.50 ± 0.19
25mg*	3.10 ± 2.53	2.81 ± 1.27

A dose-dependent increase in background fluorescence in the single-dose cohorts was observed using both fluorescence intensities of the tissue ROI of the fluorescence images (10 mg vs. 25 mg: $p=0.0006$, 25 mg vs. 50 mg: $p<0.0001$) and quantification of intrinsic fluorescence values (10 mg vs. 25 mg: $p<0.0001$, 25 mg vs. 50 mg: $p<0.0001$), whereas this remained consistently low in the cohorts pre-dosed with unlabeled cetuximab, without a significant difference between both cohorts ($p=0.4320$ and $p=0.6808$ for fluorescence imaging and MDSFR/SFF spectroscopy respectively). Median intrinsic fluorescence values in non-tumor tissue were significantly lower in the 75 + 25 mg cohort compared to the 25 mg single-dose cohort (0.0101 mm^{-1} vs. 0.0131 mm^{-1} , $p=0.0096$, respectively).

Fluorescence intensities of tumor tissue increased with increasing doses in the single dose cohorts (10 mg vs. 25 mg: $p=0.0044$, 25 mg vs. 50 mg: $p<0.0001$), but not in the in the pre-dose cohorts (75 + 15 mg vs. 75 + 25 mg: $p=0.8959$) using tissue fluorescence of the ROI of tissue slices. Quantification of intrinsic fluorescence values by MDSFR/SFF spectroscopy showed no dose-dependent increase (10 mg vs. 25 mg: $p=0.8291$, 25 mg vs. 50 mg: $p=0.2351$, 75 + 15 mg vs. 75 + 25mg: $p=0.7573$).

After correcting *ex vivo* fluorescence measurements obtained by MDSFR/SFF spectroscopy for tissue optical properties, the intrinsic fluorescence values (i.e. corrected) decreased in the majority of patients compared to the uncorrected values. Scattering and absorption coefficients mainly affected non-tumor tissue measurements (Figure S2), resulting in higher, although not significant, TBRs when comparing MDSFR/SFF spectroscopy TBRs to fluorescence imaging TBRs (2.50 ± 1.00 vs. 2.13 ± 1.04 respectively; $p=0.9780$, Figure S3).

EGFR immunohistochemistry showed expression in 96% of the tumors, in contrast to deeper seated non-tumor tissue (e.g. fat, connective tissue and muscle tissue; N=27 tissue sections; Figure S4). In addition, nineteen tissue sections contained salivary glands, of which nine (47%) showed a moderate EGFR expression. To further validate cetuximab-800CW binding specificity, fluorescence microscopy showed cetuximab-800CW was localized around tumor cell membranes (Figure 5).

Figure 5. Cetuximab-800CW binding specificity. Cetuximab-800CW fluorescence was located around the tumor cells, indicating specific binding. A Hoechst-DAPI staining was performed to visualize cell nuclei and the FITC channel was used to discriminate between autofluorescence and cetuximab-800CW derived fluorescence on the 800 nm channel. Abbreviations: H/E: Hematoxylin and Eosin. FITC: Fluorescein isothiocyanate.

Cetuximab-800CW integrity

Cetuximab-800CW integrity was confirmed four days after administration in blood samples and tumor lysates of fresh surgical specimen samples (Figure S5).

DISCUSSION

In this study, the optimal dose of cetuximab-800CW appeared to be a dose of 15 mg cetuximab-800CW preceded by 75 mg of unlabeled cetuximab. Further increasing the cetuximab-800CW dose after pre-dosing did not significantly increase the tumor-to-background ratios.

In fluorescence guided imaging, evaluation of the resection margin status is most reliably done *ex vivo*, as previous studies of our group and others show.^{18,25,36} In addition, determining the optimal dose *ex vivo* can be done more reliable since *in vivo* imaging does not allow collection of data in a standardized manner, which can influence results.²³ In the current study, we used *ex vivo* tissue fluorescence intensities of the tissue slices of tumor and adjacent non-tumor tissue to calculate the TBR. We confirmed these outcomes with MDSFR/SFF spectroscopy by correcting for tissue optical properties, although obtaining visual information from the fluorescence images is considered to be most clinically relevant for the intraoperative evaluation of the margin status since it provides wide-field information.

Here, we studied single-dose cetuximab-800CW cohorts as well as cohorts pre-dosed with an unlabeled dose cetuximab followed by cetuximab-800CW. Although visualization of fluorescence was possible even in the lower single-doses cohorts, a clinically relevant discrimination between tumor and non-tumor tissue was obtained in the pre-dosed cohorts (Figure S6). Here, background fluorescence remained consistently low, while a

dose-dependent increase was observed with in the single-dose cohorts, indicating off-target receptor saturation when pre-dosing.

A previous study using 25 mg/m² cetuximab-800CW showed that pre-dosing with 100 mg unlabeled cetuximab improves tumor visualization over 10 mg unlabeled cetuximab.³¹ Another study using panitumumab-800CW showed no difference between pre-dosed and single dose cohorts.³⁷ In the current study, we used a two-to-threefold lower cetuximab-800CW dose than previously described, which might decrease the risk of toxicity and costs. Although it is likely that the total dose of cetuximab (i.e. 90 mg in the optimal dose cohort) is relevant for potential toxicities, this is still considerably lower than previously described.^{15,31} Importantly, the lower dose of cetuximab-800CW did not influence our imaging results, because we used a highly sensitive fluorescence camera system optimized for IRDye800CW visualization, whereas Rosenthal et al. used a system optimized for Indocyanine Green visualization (peak absorption and emission 806 nm and 830 nm respectively).³¹

In addition, this study demonstrates the added value of quantification of intrinsic fluorescence values through correcting for tissue scattering and absorption coefficients for verifying the optimal dose. When interpreting fluorescence imaging results, one should realize that, regardless of the biodistribution of a fluorescent tracer, fluorescence intensities are influenced by several factors, such as the tissue optical properties, ambient light, imaging distance and the sensitivity and dynamic range of the fluorescence camera.^{23,29,38,39} Previous studies investigating EGFR-targeted imaging in HNC surgery solely used fluorescence visualization to report outcome parameters^{15,22}, although recently techniques have been described to quantify fluorescence.⁴⁰ Here, we demonstrate the added value of MDSFR/SFF spectroscopy in dose-finding studies, as we show the uncorrected fluorescence results differ significantly from corrected fluorescence values.

The preliminary sensitivity and specificity of 100% and 91% respectively clearly demonstrate the potential added value of back-table FGI of the resection specimen for intraoperative clinical decision making, despite our limited patient sample. All tumor-positive resection margins had initially been missed by visual and tactile inspection. In the current study, which involved fifteen patients, in four cases a tumor-positive resection margin was detected (4/15, 27%). There was no particular tumor-phenotypic feature that contributed to the missed positive margins. In fact, all tumors were moderately differentiated squamous cell carcinomas ranging from T1 to T4 stages. Although we observed one false-positive fluorescent lesion on the resection margin, a high sensitivity is crucial in HNC treatment, as leaving residual tumor negatively influences treatment outcome and survival.⁴ Back-table FGI serves as a 'red-flag' imaging technique that allows the surgeon to perform more targeted fresh frozen section analysis or to perform a re-resection while the patient is still anesthetized. This potentially prevents second surgery or (extensive) adjuvant treatment regimens. This is congruent with data from previous studies investigating the added value of back-table FGI.^{16,18} The benefit of a specimen-driven approach for margin evaluation is that it can be performed in a controlled and standardized environment, which overcomes previously described factors that influence fluorescence imaging results.²³

In the current study, we showed that it was feasible to perform FGI using a flexible nasendoscope which allowed imaging of locations that were difficult to assess using an open-surgery fluorescence camera. Although the fiber optic approach reduced imaging resolution and detection sensitivity compared to the wide-field fluorescence imaging system, we believe the nasendoscopic system can assist in detecting undisclosed lesions in the upper aerodigestive tract.

EGFR was validated as a target using immunohistochemistry. Although we observed a heterogeneous EGFR expression in tumor tissue microscopically, this did not influence macroscopic fluorescence imaging results. In the current study, 96% of HNSCC tissue sections expressed EGFR. Although the normal mucosa also expressed EGFR, this was not observed in the muscle, fat and connective tissue that forms the basal resection margin, where 80% of tumor-positive resection margins are located.⁹ Although we observed EGFR expression in salivary glands which is in line with literature, this did not influence the evaluation of resection margins in the current study.

In conclusion, we found that a dose of 75 mg unlabeled cetuximab followed by 15 mg cetuximab-800CW was well tolerated and allowed optimal discrimination between tumor and non-tumor tissue. MDSFR/SFF spectroscopy verifies the localization and quantifies the concentration of cetuximab-800CW in tumor and non-tumor tissue by correcting for tissue optical properties and provides important information on the tracer biodistribution, which supports our rationale for the optimal dose. The results of this study have led to the initiation of a currently ongoing phase-II clinical study in our institute (NCT03134846), investigating the clinical value of back-table FGI using cetuximab-800CW in a larger population of HNSCC patients.

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COMPETING INTERESTS

GMvD is member of the Scientific Advisory Board of SurgVision BV, and CEO, founder and shareholder of TRACER Europe BV / AxelarX. GMvD and WBN received an unrestricted research grant from SurgVision BV.

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REFERENCES

- 1 Jemal A, Bray F, Center, Melissa M, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin.* 2011; 61: 69–90.
- 2 Baumeister P, Baumuller K, Harreus U, Reiter M, Welz C. Evaluation of margins in head and neck squamous cell carcinoma from the surgeon's perspective. *Head Neck.* 2018; 40: 963–972.
- 3 Orosco RK, Tapia VJ, Califano JA, Clary B, Cohen EEW, Kane C, et al. Positive Surgical Margins in the 10 Most Common Solid Cancers. *Sci Rep.* 2018; 8: 5686.
- 4 Sutton DN, Brown JS, Rogers SN, Vaughan ED, Woolgar JA. The prognostic implications of the surgical margin in oral squamous cell carcinoma. *Int J Oral Maxillofac Surg* 2003; 32: 30–34.
- 5 Binahmed A, Nason RW, Abdoh AA. The clinical significance of the positive surgical margin in oral cancer. *Oral Oncol.* 2007; 43: 780–784.
- 6 Aliperti LA, Predina JD, Vachani A, Singhal S. Local and systemic recurrence is the Achilles heel of cancer surgery. *Ann Surg Oncol* 2011; 18: 603–607.
- 7 McMahon J, O'Brien CJ, Pathak I, Hamill R, McNeil E, Hammersley N, et al. Influence of condition of surgical margins on local recurrence and disease-specific survival in oral and oropharyngeal cancer. *Br J Oral Maxillofac Surg.* 2003; 41: 224–231.
- 8 Wong LS, McMahon J, Devine J, McLellan D, Thompson E, Farrow A, et al. Influence of close resection margins on local recurrence and disease-specific survival in oral and oropharyngeal carcinoma. *Br J Oral Maxillofac Surg.* 2012; 50: 102–108.
- 9 Woolgar JA, Triantafyllou A. A histopathological appraisal of surgical margins in oral and oropharyngeal cancer resection specimens. *Oral Oncol* 2005; 41: 1034–1043.
- 10 Gephardt GN, Zarbo RJ: Interinstitutional comparison of frozen section consultations. A college of American Pathologists Q-Probes study of 90,538 cases in 461 institutions. *Arch Pathol Lab Med.* 1996; 120: 804–809.
- 11 Gao RW, Teraphongphom N, de Boer E, van den Berg NS, Divi V, Kaplan MJ, et al. Safety of panitumumab-IRDye800CW and cetuximab-IRDye800CW for fluorescence-guided surgical navigation in head and neck cancers. *Theranostics.* 2018; 8: 2488–2495.
- 12 Hartmans E, Tjalma JJJ, Linssen MD, Allende PBG, Koller M, Jorritsma-Smit A, et al. Potential Red-Flag Identification of Colorectal Adenomas with Wide-Field Fluorescence Molecular Endoscopy. *Theranostics.* 2018; 8: 1458–1467.
- 13 van Dam GM, Themelis G, Crane LMA, Harlaar NJ, Pleijhuis RG, Kelder W, et al. Intraoperative tumor-specific fluorescence imaging in ovarian cancer by folate receptor-alpha targeting: first in-human results. *Nat Med.* 2011; 17: 1315–1319.
- 14 Burggraaf J, Kamerling IMC, Gordon PB, Schrier L, de Kam ML, Kales AJ, et al. Detection of colorectal polyps in humans using an intravenously administered fluorescent peptide targeted against c-Met. *Nat Med.* 2015; 21: 955–961.
- 15 Rosenthal EL, Warram JM, de Boer E, Chung TK, Korb ML, Brandwein-Gensler M, et al. Safety and Tumor Specificity of Cetuximab-IRDye800 for Surgical Navigation in Head and Neck Cancer. *Clin Cancer Res.* 2015; 21: 3658–3666.
- 16 Harlaar NJ, Koller M, de Jongh SJ, van Leeuwen BL, Hemmer PH, Kruijff S, et al. Molecular fluorescence-guided surgery of peritoneal carcinomatosis of colorectal origin: a single-centre feasibility study. *Lancet Gastroenterol Hepatol.* 2016; 1: 283–290.
- 17 Tummers WS, Miller SE, Teraphongphom NT, Gomez A, Steinberg I, Huland DM, et al. Intraoperative Pancreatic Cancer Detection using Tumor-Specific Multimodality Molecular Imaging. *Ann Surg Oncol.* 2018; 25: 1880–1888.
- 18 Koller M, Qiu S-Q, Linssen MD, Jansen L, Kelder W, de Vries J, et al. Implementation and benchmarking of a novel analytical framework to clinically evaluate tumor-specific fluorescent tracers. *Nat Commun.* 2018; 9: 37–39.
- 19 Lamberts LE, Koch M, de Jong JS, Adams ALL, Glatz J, Kranendonk MEG, et al. Tumor-Specific Uptake of Fluorescent Bevacizumab-IRDye800CW Microdosing in Patients with Primary Breast Cancer: A Phase I Feasibility Study. *Clin Cancer Res.* 2017; 23: 2730–2741.
- 20 Ang KK, Berkey BA, Tu X, Zhang H-Z, Katz R, Hammond EH, et al. Impact of epidermal growth factor receptor expression on survival and pattern of relapse in patients with advanced head and neck carcinoma. *Cancer Res.* 2002; 62: 7350–7356.
- 21 Grandis JR, Tweardy DJ. Elevated levels of transforming growth factor alpha and epidermal

- markers of carcinogenesis in head and neck cancer. *Cancer Res.* 1993; 53: 3579–3584.
- 22 Gao RW, Teraphongphom NT, van den Berg NS, Martin BA, Oberhelman NJ, Divi V, et al. Determination of Tumor Margins with Surgical Specimen Mapping Using Near-Infrared Fluorescence. *Cancer Res.* 2018; 78: 5144–5154.
 - 23 Koch M, Symvoulidis P, Ntziachristos V. Tackling standardization in fluorescence molecular imaging. *Nature Photonics.* 2018; 12: 505–515.
 - 24 Helmchen F, Denk W. Deep tissue two-photon microscopy. *Nat Methods.* 2005; 2: 932–940.
 - 25 van Keulen S, van den Berg NS, Nishio N, Birkeland A, Zhou Q, Lu G, et al. Rapid, non-invasive fluorescence margin assessment: Optical specimen mapping in oral squamous cell carcinoma. *Oral Oncol.* 2019; 88: 58–65.
 - 26 van Keulen S, Nishio N, Birkeland A, Fakurnejad S, Martin B, Forouzanfar T, et al. The Sentinel Margin: Intraoperative Ex Vivo Specimen Mapping Using Relative Fluorescence Intensity. *Clin Cancer Res.* 2019; 25: 4656–4662.
 - 27 Middelburg TA, Hoy CL, Neumann HAM, Amelink A, Robinson DJ. Correction for tissue optical properties enables quantitative skin fluorescence measurements using multi-diameter single fiber reflectance spectroscopy. *J Dermatol Sci.* 2015; 79: 64–73.
 - 28 van Leeuwen-van Zaane F, Gamm UA, van Driel PBAA, Snoeks TJ, de Bruijn HS, van der Ploeg-van den Heuvel A, et al. Intrinsic photosensitizer fluorescence measured using multi-diameter single-fiber spectroscopy in vivo. *J Biomed Opt.* 2014; 19: 15010.
 - 29 Kanick SC, Robinson DJ, Sterenborg HJCM, Amelink A. Extraction of intrinsic fluorescence from single fiber fluorescence measurements on a turbid medium. *Opt Lett.* 2012; 37: 948–950.
 - 30 Nagengast WB, Hartmans E, Garcia-Allende PB, Peters FTM, Linssen MD, Koch M, et al. Near- infrared fluorescence molecular endoscopy detects dysplastic oesophageal lesions using topical and systemic tracer of vascular endothelial growth factor A. *Gut.* 2019; 68: 7–10.
 - 31 Moore LS, Rosenthal EL, de Boer E, Prince AC, Patel N, Richman JM, et al. Effects of an Unlabeled Loading Dose on Tumor-Specific Uptake of a Fluorescently Labeled Antibody for Optical Surgical Navigation. *Mol Imaging Biol.* 2016; 19: 610–616.
 - 32 Linssen MD, Weeie Ter EJ, Allersma DP, Lub-de Hooge MN, van Dam GM, Jorritsma-Smit A, et al. Roadmap for the Development and Clinical Translation of Optical Tracers Cetuximab-800CW and Trastuzumab-800CW. *J Nucl Med.* 2019; 60: 418–423.
 - 33 [Internet] Helliwell T, Woolgar JA. Dataset for histopathology reporting of mucosal malignancies of the oral cavity. The royal college of pathologists 2013. <https://www.rcpath.org/uploads/assets/c4a9faf7-393a-4ba8-9532f719d8cdff3b/Dataset-for-histopathology-reporting-of-mucosal-malignancies-of-the-oral-cavity.pdf>
 - 34 van Leeuwen-van Zaane F, Gamm UA, Van Driel PBAA, Snoeks TJA, de Bruijn HS, van der Ploeg van den Heuvel A, et al. In vivo quantification of the scattering properties of tissue using multi-diameter single fiber reflectance spectroscopy. *Biomed Opt Express.* 2013; 4: 696–13.
 - 35 de Boer E, Warram JM, Tucker MD, Hartman YE, Moore LS, de Jong JS, et al. In Vivo Fluorescence Immunohistochemistry: Localization of Fluorescently Labeled Cetuximab in Squamous Cell Carcinomas. *Sci Rep.* 2015; 5: 10169.
 - 36 de Jongh SJ, Tjalma JJJ, Koller M, Linssen MD, Vonk J, Dobosz M, et al. Back-table fluorescence-guided imaging for circumferential resection margin evaluation in locally advanced rectal cancer patients using bevacizumab-800CW. *J Nucl Med.* 2019; DOI: 10.2967/jnumed.119.232355
 - 37 Nishio N, van den Berg NS, van Keulen S, Martin BA, Fakurnejad S, Zhou Q, et al. Optimal Dosing Strategy for Fluorescence-Guided Surgery with Panitumumab-IRDye800CW in Head and Neck Cancer. *Mol Imaging Biol.* 2019; DOI: 10.1007/s11307-019-01358-x
 - 38 Koch M, Ntziachristos V. Advancing Surgical Vision with Fluorescence Imaging. *Annu Rev Med.* 2016; 67: 153–164.
 - 39 de Visscher SAHJ, Witjes MJH, Kascakova S, Sterenborg HJCM, Robinson DJ, Roodenburg JLN, et al. In vivo quantification of photosensitizer concentration using fluorescence differential path-length spectroscopy: influence of photosensitizer formulation and tissue location. *J Biomed Opt.* 2012; 17: 067001.
 - 40 Nishio N, van Keulen S, van den Berg NS, Lu G, LaRochelle EP, Davis SC, et al. Probe-based fluorescence dosimetry of an antibody-dye conjugate to identify head and neck cancer as a first step to fluorescence-guided tissue preselection for pathological assessment. *Head Neck.* 2019; DOI: 10.1002/hed.25964

SUPPLEMENTAL MATERIAL

Table S1. Adverse events related to tracer administration. Six possible related adverse occurred due to cetuximab-800CW administration. *The bronchospasm occurred after accidentally too rapid infusion (10x higher than planned).

	Adverse Event	Grade	Intervention
1	Hypotension (during surgery)	I	I.V. fluid suppletion
2	Temporary QTc prolongation	I	None
3	Temporary QTc prolongation	I	None
4	Flush	I	None
5	Fever	I	None
6	Bronchospasm*	II	2 mg Clemastine I.V. + 4 mg Dexamethosaon I.V.

Table S2. Cetuximab-800CW concentrations based on MDSFR/SFF spectroscopy measurements in tissue slices. Increasing single doses of cetuximab-800CW show increasing background fluorescence in non-tumor tissue whereas background fluorescence remains low when an unlabeled pre-dose of cetuximab is administered one hour prior to cetuximab-800CW. Values depicted as median $\pm$ IQR. *75 mg cetuximab is administered one hour prior to cetuximab-800CW administration.

Dose-cohort	Concentration in tumor (nmol/mL)	Concentration in non-tumor tissue (nmol/mL)	Ratio
10 mg	4.78 $\pm$ 0.36	1.67 $\pm$ 0.41	2.86
25 mg	5.45 $\pm$ 2.62	2.35 $\pm$ 0.77	2.32
50 mg	6.02 $\pm$ 1.46	3.37 $\pm$ 0.89	1.79
15 mg*	4.50 $\pm$ 0.59	1.93 $\pm$ 0.22	2.33
25 mg*	4.70 $\pm$ 1.22	1.70 $\pm$ 0.42	2.81

Figure S1. Back-table fluorescence-guided imaging of a fluorescence-positive but tumor-negative resection margin. Excised specimen (partial tongue resection) which shows fluorescence at the primary tumor (mucosal side). At the caudal resection margin, a localized fluorescent lesion (yellow arrow) that corresponds to tumor-negative resection margin of 2 mm (caudal resection surface). Tumor is delineated with a solid black line. Abbreviations: H/E: Hematoxylin & Eosin.

Figure S2. Influence of tissue scattering and absorption on intrinsic fluorescence values during FGI. The intrinsic fluorescence values (i.e. corrected for scattering and absorption) are depicted as a percentage of the uncorrected fluorescence values acquired with MDSFR/SFF spectroscopy direct contact measurements. Non-tumor tissue is more affected by tissue optical properties compared to tumor tissue. Bars represent median values with interquartile range.

Figure S3. Difference in TBR between quantification of intrinsic fluorescence values (MDSFR/SFF spectroscopy) versus fluorescence visualization (camera). Dots represent the median TBRs values of the in vivo intrinsic fluorescence values measured using MDSFR/SFF spectroscopy (right) and fluorescence visualization using the fluorescence camera system (left) per patient. Error bars represent median values and interquartile range.

Figure S4. Epidermal Growth Factor Receptor (EGFR) membrane expression. Muscle, fat and connective tissue showed no EGFR expression, whereas a clear expression was observed in tumor tissue in 96% of the tissue sections (N=27). Normal mucosa showed in the majority of cases mild (1+) EGFR expression. A representative image of each EGFR staining intensity is depicted.

Figure S5. Cetuximab-IRDye800CW integrity. Representative examples of cetuximab-800CW integrity ($\pm$ 151kD). **A:** SDS-PAGE of a blood sample collected four days after tracer administration. **B:** SDS-PAGE of a fresh-frozen tumor lysates collected immediately after tumor excision. *75 mg cetuximab is administered one hour prior to cetuximab-800CW administration.

Figure S6. Representative tissue slices containing tumor per dosing cohort. Representative images of tissue slices containing both tumor and non-tumor tissue per dose cohort. Histological proven tumor tissue is delineated with a dotted white line. Note the enhanced discrimination between tumor and non-tumor tissue and sharp delineation in the pre-dosed cohorts. Fluorescence intensities are scaled from minimum to maximum. *75 mg cetuximab is administered one hour prior to cetuximab-800CW administration. CTX: Cetuximab.

Supplemental video

Video S1. Intraoperative visualization of a tongue carcinoma. Intraoperative visualization of cetuximab-800CW fluorescence of lateral tongue carcinoma. Patient is dosed four days prior to surgery with 75 mg cetuximab followed by 15 mg cetuximab-800CW.

**Back-table fluorescence-guided
imaging for evaluation of
circumferential resection margins
in locally advanced rectal cancer
patients using bevacizumab-800CW**

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ABSTRACT

Rationale

Negative circumferential resection margins (CRM) are the cornerstone for curative treatment of locally advanced rectal cancer (LARC). However, in up to 18.6% of patients' tumor-positive resection margins are detected upon histopathology. In this proof-of-concept study, we investigated the feasibility of optical molecular imaging as a tool to evaluate the CRM directly after surgical resection in order to improve tumor-negative CRM rates.

Methods

LARC patients that were treated with neoadjuvant chemoradiotherapy received an intravenous bolus injection of 4.5 mg bevacizumab-800CW, a fluorescent tracer targeting vascular endothelial growth factor A (VEGFA), 2-3 days before surgery (NCT01972373). To evaluate the CRM status, back-table fluorescence-guided imaging (FGI) was performed of the fresh surgical resection specimens (N = 8). These results were correlated to histopathology. Secondly, to determine the sensitivity and specificity of bevacizumab-800CW for tumor detection, a mean fluorescence intensity (MFI) cut-off value was determined on the formalin-fixed tissue slices (N = 42; 17 patients). Local bevacizumab-800CW accumulation was evaluated by fluorescence microscopy.

Results

Back-table FGI correctly identified a tumor-positive CRM by high fluorescence intensities in one of two patients (50%) with a tumor-positive CRM. The other patient showed low fluorescence intensities, although (sub-)millimeter tumor deposits were present <1 mm of the CRM. FGI correctly identified 5/6 tumor-negative CRMs (83%). The one patient with false-positive findings had a marginal negative CRM of only 1.4 mm. ROC analysis of fluorescence intensities of formalin-fixed tissue slices gave an optimal MFI cut-off value for tumor detection of 5,775 (sensitivity and specificity of 96.16% and 80.39% respectively). Bevacizumab-800CW enabled a clear differentiation between tumor and normal tissue up to a microscopic level, with a tumor-to-background ratio of 4.7 ± 2.5 (mean $\pm$ SD).

Conclusion

In this proof-of-concept study, we showed the potential of back-table FGI to evaluate the CRM status in LARC patients. Optimization of this technique with adaptation of standard operating procedures could change perioperative decision-making with regard to extending resections or applying intraoperative radiation therapy in case of positive CRMs.

INTRODUCTION

In today's clinical practice, patients with locally advanced rectal cancer (LARC) receive long-course neoadjuvant chemoradiotherapy (nCRT) followed by surgical resection using the total mesorectal excision (TME) principles. nCRT induces tumor downsizing and downstaging, which facilitates complete resection by TME, resulting in significantly reduced local recurrence rates and increased opportunity for sphincter-sparing resections.¹⁻⁵

Obtaining negative circumferential resection margins (CRM) is key in rectal cancer therapy. The CRM has proven to be one of the most important predictors for local recurrence and to a lesser extent the development of distant metastases and survival.⁶ Restaging after nCRT occurs through a high-resolution MRI-scan for tumor staging and a CT-scan of thorax and abdomen for distant metastasis and lymph node staging.⁷⁻⁹ Accurate restaging can be highly challenging as differentiating between desmoplastic reaction and viable tumor tissue on preoperative imaging modalities is often difficult after nCRT.

Intraoperatively, surgeons mainly rely on visual and tactile inspection for margin assessment and differentiation between tumor and healthy tissue. This is often inaccurate, especially after nCRT, as small tumor deposits are frequently present within fibrotic parts.¹⁰ When in doubt, resection margins can be evaluated intraoperatively by frozen section pathologic evaluation, but this is time-consuming, costly and poses a high risk of sampling error.¹¹

Despite nCRT, TME-surgery and frozen section analysis, a tumor-positive CRM, defined as tumor presence ≤ 1 mm from the CRM, is detected in up to 18.6% of primary LARC surgeries upon final histopathology.⁷⁻⁹ Peri-operative resection margin evaluation using optical molecular imaging could improve negative CRM rates by extending resections, applying intraoperative radiation therapy (IORT) or more innovative treatment modalities like photo-immunotherapy of the wound bed.¹² In contrast, when a margin is evaluated to be tumor-negative, extended resections could be avoided.

The aim of this proof-of-concept study was to evaluate if back-table fluorescence-guided imaging (FGI) using the near-infrared fluorescent tracer bevacizumab-800CW, could aid in evaluating the CRM status at the surgical theater. Bevacizumab-800CW targets vascular endothelial growth factor A (VEGFA), which is overexpressed in LARC as well as many other solid tumors.¹³⁻¹⁵ We retrospectively analyzed back-table FGI data of fresh surgical specimens of LARC patients that were treated with nCRT. This technique may eventually allow real-time determination of CRM during surgery, which could aid intraoperative clinical decision-making with regard to extending resections or applying IORT in case of a positive CRM, to improve the outcome of LARC patients.

MATERIAL AND METHODS

Study design and population

Postoperative fluorescence imaging data were collected from 25 LARC patients enrolled in a clinical trial evaluating VEGFA-targeted fluorescence molecular endoscopy (Clinicaltrials.gov identifier: NCT01972373). Eligibility criteria included histologically proven LARC, with the inferior margin within 16 cm from the anal verge and treatment with long-course nCRT. To determine if back-table FGI could aid in evaluating the CRM status, patients were

included if fluorescence imaging data was available from at least the anterior and posterior sides of the fresh surgical specimen. Furthermore, to evaluate local bevacizumab-800CW accumulation and determine the sensitivity and specificity of bevacizumab-800CW for tumor detection using a fluorescence cut-off value, patients with high-resolution fluorescence images available of formalin-fixed tissue slices were included. The study was performed at the University Medical Center Groningen, approved by the Institutional Review Board (METc 2013/067) and all subjects signed a written informed consent.

Surgery

After nCRT, all patients received an intended curative resection by either low anterior resection (LAR) for proximal rectum tumors or abdominal perineal resection (APR) for distal rectum tumors. Resections were extended outside the TME planes in case of tumor growth into adjacent organs. Patients only received IORT if judged necessary based on preoperative suspicion of mesorectal fascia involvement or intraoperative evaluation by the surgeons.

Histopathological processing

Histopathological processing of surgical specimens was performed by a board-certified gastrointestinal pathologist. After gross pathology, CRMs were inked black and staple lines were removed. Specimens were anteriorly opened from proximal to distal, except for specimens with an anterior lesion, which were opened until the rectal fold. All specimens were formalin-fixed for at least 48 hours and serially sliced perpendicular to the rectum, from distal to proximal into ± 0.5 cm thick tissue slices. Distal and proximal resection surfaces plus additional areas of interest (i.e. regions suspect for CRM involvement, perineural growth, vascular invasion, lymph nodes, etc.) were included for paraffin embedding. Formalin-fixed paraffin-embedded (FFPE) tissue blocks were cut in 4 μ m tissue sections and hematoxylin eosin (HE) stained for routine histopathological examination. Immunohistochemistry was performed if required. A tumor-positive CRM was defined as tumor presence ≤ 1 mm from the inked CRM, in accordance with the Dutch national guidelines.

Figure 1. Schematic overview of study design. Bevacizumab-800CW (4.5 mg) was administered intravenously two to three days prior to surgery. Fluorescence-guided imaging was performed at every step during pathological processing: back-table of the fresh surgical specimen, of the tissue slices and tissue sections. Results were correlated to histopathology.

Bevacizumab-800CW

All patients received a 4.5mg IV bolus injection of bevacizumab-800CW (1 mg/ml) two-to-three days before surgery based on microdosing regulations (Figure 1). Bevacizumab-800CW was produced under cGMP conditions at the University Medical Center Groningen, as described previously.¹⁶

Back-table FGI

To evaluate the CRM status, back-table FGI of the fresh surgical specimen was performed in a light-tight room directly after surgery using the Explorer Air fluorescence camera (SurgVision BV, Groningen, The Netherlands, Figure 1). Areas with high fluorescence signals were marked with a pin and subsequently inked with a different color from the CRM during pathological processing, to ensure an accurate correlation of fluorescence with histopathology.

Fluorescence imaging of tissue slices and sections

To evaluate the local tracer accumulation, fluorescence imaging of both sides of all formalin-fixed tissue slices was performed using the Explorer Vault, a standardized and light-tight fluorescence imaging system (SurgVision BV, Groningen, The Netherlands). Thereafter, two-to-three tissue slices containing tumor and/or normal rectal tissue were imaged per patient using the high-resolution Odyssey CLx fluorescence imaging system (LI-COR Biosciences Inc., Lincoln, NE; Figure 1). Additional areas of interest based on fluorescence imaging were also paraffin embedded, sliced in 4 μ m tissue sections and HE stained. All FFPE tissue blocks were fluorescently scanned using the Odyssey CLx (Figure 1). For comparison purposes, all fluorescence images were thresholded to minimum-maximum values per imaging modality for each individual patient.

To broaden our understanding of the overall penetration, distribution, and accumulation of bevacizumab-800W in rectal cancer tissue, a combination of optical tissue clearing and light-sheet fluorescence microscopy (LSFM) was performed on selected tissue slices, as previously described for preclinical tissue samples (Supplemental Material).¹⁷

Fluorescence grid analysis

Finally, to evaluate the sensitivity and specificity of bevacizumab-800CW for tumor detection, a fluorescence grid analysis was used based on histopathology as a gold standard to determine a fluorescence cut-off value (Supplemental Material).

Statistical analysis

Normally distributed data is presented as mean values with standard deviation (SD) and skewed data as median values with interquartile range (IQR). A receiver operating characteristics (ROC) curve was plotted to determine the MFI cut-off value for tumor detection. P-values <0.05 were considered statistically significant. Statistical analyses were performed using Prism (version 7.0, GraphPad Software).

RESULTS

Patient characteristics

In this retrospective proof-of-concept study, eight of 25 patients met the criteria to determine the feasibility of back-table FGI for the evaluation of the CRM status (Supplemental Figure 1). For the second aim of this study, to evaluate local bevacizumab-800CW distribution and determine the sensitivity and specificity of bevacizumab-800CW for tumor detection on high-resolution tissue slices, 17/25 patients met the inclusion criteria (Supplemental Figure 1). Patient characteristics are depicted in Table 1. All patients received 4.5 mg bevacizumab-800CW IV, 2-3 days prior to surgery. Ten patients underwent a LAR and seven patients an APR. There were no tracer-related (serious) adverse events in any of the patients.

Evaluation of the CRM status

Of eight evaluated cases, two patients (25%) presented with a tumor-positive CRM on final histopathology. Back-table FGI correctly predicted the tumor-positive CRM in one of these patients due to increased fluorescence signals in the CRM (50%, Figure 2). This patient was treated with an APR with en-bloc resection of the sacrum and a wide perineal excision because of a fistula. Intraoperatively, two frozen section analyses were performed of the lateral resection surface, both of which were benign. Interestingly, during back-table FGI of the fresh surgical specimen, high fluorescence signals were observed at the lateral resection margin (Figure 2A). Fluorescence imaging of the corresponding tissue slice also showed high fluorescence that seemed to reach into the CRM, while the surrounding non-tumor tissue showed low fluorescence (Figure 2B). Final histopathology proved that this exact location was a tumor-positive CRM (Figure 2C; orange arrows).

Table 1. Patient and tumor characteristics. nCRT = neoadjuvant chemoradiotherapy; pCR = pathological complete response; TNM = Tumor, Node, Metastasis.

	CRM evaluation (N = 8)		Fluorescence cut-off value (N = 17)	
	No.	%	No.	%
Sex				
Male	5	62.5%	12	70.6%
Female	3	37.5%	5	29.4%
Age (years)				
Median (range)	56 (54 - 61)		56 (31 - 76)	
Duration between nCRT - Surgery (days)				
Median (interquartile range)	87 (76-111)		87 (77-117)	
Surgery				
Low-anterior resection	5	62.5%	10	58.8%
- Including adjacent organs	1	-	2	-
Abdominoperineal resection	3	37.5%	7	41.2%
- Including adjacent organs	3	-	5	-
Intraoperative radiation therapy				
Not standby	3	37.5%	-	-
Standby	4	50%		
Applied	1	12.5%		
Histopathological staging				
pT0 N0 M0 (pCR)	1	12.5%	1	5.9%
pT2 N0 M0	2	25.0%	2	11.8%
pT3 N0 M0	1	12.5%	4	23.5%
pT3 N1 M0	0	-	3	17.6%
pT3 N2 M0	2	25.0%	5	29.4%
pT4 N0 M0	2	25.0%	2	11.8%
Circumferential resection margin (CRM)				
≤ 1 mm (tumor-positive)	2	25.0%	3	17.6%
1-2 mm	1	12.5%	2	11.8%
> 2 mm	4	50.0%	11	64.7%
pCR	1	12.5%	1	5.9%
Distal resection margin				
≤ 1 mm (tumor-positive)	0	-	0	-
1-2 mm	0	-	1	5.9%
> 2 mm	7	87.5%	15	88.2%
pCR	1	12.5%	1	5.9%

Figure 2. Back-table FGI of a patient with a tumor-positive CRM, with a black pin at the location showing increased fluorescence, to enable an accurate correlation with histology (A, orange arrow). Fluorescence imaging of two corresponding tissue slices (B) and further microscopic fluorescence imaging and histological correlation (C), with orange arrows indicating the location of the tumor-positive CRM. High fluorescence is observed at the CRM of tissue slice 1 containing the tumor-positive CRM, whereas low fluorescence was observed in the non-tumor tissue slice 2, corresponding to the microscopy results. FFPE = formalin-fixed paraffin embedded; HE = hematoxylin eosin; N = non-tumor; T = tumor.

The second patient with a tumor-positive CRM received a LAR. Back-table FGI of the fresh surgical specimen and subsequent fluorescence imaging of the tissue slices showed low fluorescence intensities in the CRM, apart from fluorescence in two enlarged suspicious lymph nodes that proved to be tumor-positive (Supplemental Figure 2A). However, the pathologist reported a tumor-positive CRM that was solely based on the presence of isolated microscopic vital-looking tumor deposits of (sub-)millimeter size within a distance of 0.2-1 mm of the CRM (Supplemental Figure 2B; orange arrow). The tumor volume was limited due to the (sub-)millimeter size of the tumor deposits, which may explain the

negative imaging results.

Six out of 8 patients (75%) had a tumor-negative CRM, of which five were correctly predicted by low fluorescence intensities during back-table FGI (representative example in Supplemental Figure 3). Subsequent fluorescence imaging of tissue slices also showed low fluorescence in relation to the CRM, although fluorescence was observed in the (intra) luminal tumor, except for the patient with a pathological complete response. IORT was applied to one of these patients due to a macroscopic suspicion of a tumor-positive CRM, while histopathology showed a tumor-negative CRM (>1 cm).

The remaining patient with a tumor-negative CRM received a low-anterior resection with en-bloc resection of the uterus, cervix, adnexa and the distal right ureter due to tumor ingrowth. Increased fluorescence was observed at the cervix during back-table FGI and subsequent fluorescence imaging of the tissue slices, potentially indicating a tumor-positive CRM. Although the fluorescence colocalized with a tumor deposit upon histopathology, the CRM was defined as tumor-negative, as the distance to the CRM was 1.4 mm.

Figure 3. Fluorescence grid analysis. All tumor tissue was delineated on a hematoxylin eosin (HE)-staining (A) and subsequently overlaid on the high-resolution fluorescence images of tissue slices (B). Each 3-by-3 mm square was selected as tumor-positive (C, orange) or tumor-negative (C, green). Median fluorescence intensities (MFI) were calculated per square, with significantly higher fluorescence in tumor tissue compared to surrounding non-tumor tissue (12,000 versus 2,140; $P < 0.001$; D). Individual areas under the curve were determined per patient (E) and a receiver operating characteristic (ROC)-curve was plotted, showing an area under the curve (AUC) of 0.94 (F).

Fluorescence cut-off value and local bevacizumab-800CW accumulation

High-resolution fluorescence Odyssey scans of formalin-fixed tissue slices ($n = 42$) available from 17 patients were used to determine a semi-quantitative fluorescence cut-off value for tumor detection. A total of 5,101 grid-squares were analyzed, of which 446 were classified as tumor-positive and 4,655 as tumor-negative (Figures. 3A-3C). Significantly higher fluorescence intensities were observed in tumor areas (median MFI: 12,000) compared to surrounding non-tumor tissue (median MFI: 2,140; $P < 0.0001$; Figure 3D), which resulted in a ratio between tumor-to-surrounding tissue of 4.7 ± 2.5 (mean $\pm$ SD). Receiver operating characteristics (ROC) curves were plotted per patient (Figure 3E) and for all patients combined, with an area under the curve of 0.94 (std. error 0.0039; Figure 3F). In our limited sample size, an optimal MFI cut-off value of 5,775 was calculated using Youden's J statistics ($J = 0.77$), with a sensitivity and specificity of 96.19% and 80.39% respectively.

Finally, we evaluated the distribution and accumulation of bevacizumab-800CW on a microscopic level. Bevacizumab-800CW was mainly localized in the micro-environment of the tumor cells (Figure 4), which was in line with the expected location of VEGFA (Supplemental Video 1).

Figure 4. Representative example of light-sheet fluorescence microscopy to evaluate the local tracer accumulation in a rectal cancer tissue slice, showing the tissue morphology based on autofluorescence (A), the fluorescence (B), a morphology and fluorescence overlay (C) and the corresponding histology (D). Magnified images are depicted in the bottom row. Increased bevacizumab-800CW binding can be seen in the micro-environment of the tumor cells compared to surrounding normal mucosa and fibrosis.

DISCUSSION

Accurate perioperative evaluation of resection margins is highly important for the prognosis of LARC patients. In this explorative study, we demonstrate for the first time the feasibility of back-table FGI for identification of tumor-positive resection margins in LARC patients. Our data suggests that FGI may have the potential to guide current clinical decision-making with regard to additional targeted resections or application of IORT. Future studies using a standardized imaging protocol with larger patients samples should confirm these results.

Over the past decade, optical molecular imaging has predominantly been applied for intraoperative guidance and surgical navigation. Two clinical studies have demonstrated the feasibility and potential benefit of fluorescence-guided surgery in colorectal cancer patients.^{18,19} However, fluorescence-guided surgery is subject to several limitations related to the currently available hardware, such as variation in image-acquisition parameters and interference of ambient light. Moreover, homogenous tracer excitation is difficult especially in rectal cancer surgery, as the pelvic region is a confined surgical field often situated deep in the patient. Possibly, the development of fluorescence laparoscopes and robotic systems sensitive enough to detect micro-dosed fluorescent tracers may enable more reliable intraoperative evaluation of rectal cancer resection margins in the future.

Back-table FGI circumvents these limitations, as it makes use of a controlled, standardized and closed-field imaging environment that results in a consistent field-of-view, imaging distance and image-acquisition parameters.²⁰ This enables a highly sensitive and semi-quantitative evaluation of resection margins, within a maximum of one hour after specimen excision at the OR.

The potential added value of back-table FGI was clearly demonstrated by the patient in which a tumor-positive CRM was detected at the OR, while surgical assessment and frozen section analysis were false-negative. Although intraoperative frozen section analysis is recommended for low and mid rectal tumors, it is prone to sampling error, labor-intensive and significantly prolongs anesthesia.^{21,22} By 'bringing pathology to the operating theatre' using back-table FGI, both surgeons and pathologist may be guided in correctly assessing the CRM status and evaluating the need for extended surgery or application of IORT, thereby improving personalized treatment.

In rectal cancer surgery, caution is to be taken with unnecessarily extending TME surgery to a partial or total resection of adjacent pelvic organs or applying IORT, as this can result in substantial postoperative complications and is associated with the need for reinterventions.²³ Back-table FGI correctly predicted five out of six tumor-negative CRMs, which might have prevented unnecessary IORT application in one patient. In contrast, one close margin of 1.4 mm was identified as tumor-positive based on fluorescence. Although currently a tumor-positive margin is defined as tumor cells ≤ 1 mm of the CRM, this definition is under debate, as both patients with a CRM of 0.0 - 1.0 mm and 1.1 - 2.0 mm are shown to have an equally increased 2-year risk of local recurrence and distant metastases.^{8,24} Application of fluorescence may highlight the location of a (potentially) tumor-positive CRM, which allows the surgeon to perform more 'targeted' intraoperative frozen section analysis.

FGL may also support the pathologist by differentiating tumor from healthy tissue, i.e. fluorescence-guided pathology. Currently, tissue sampling is performed by gross examination of the surgical specimen and tissue slices, which can be challenging as the tissue architecture is changed by nCRT. Using the fluorescence-grid analysis, we showed a tumor-to-background ratio of 4.7 ± 2.5 on tissue slices, with a high sensitivity (96.19%) and specificity (80.39%) for tumor detection. A fluorescence cut-off value provides more objective information and may enable targeted tissue sampling, potentially saving labor, time and money. In addition, the fluorescence grid analysis can be used to evaluate tracer biodistribution and can easily be implemented on other closed-field imaging devices.

This study has several limitations. First of all, the number of patients included for CRM evaluation was relatively low, as this was a retrospective proof-of-concept analysis of a clinical trial evaluating fluorescence molecular endoscopy (NCT01972373), and imaging techniques as well as *ex vivo* imaging procedures have developed throughout the study. Secondly, a dose-escalation was not incorporated in this study design. Currently available evidence from two bevacizumab-800CW dose-escalation studies suggests that increasing the dose above microdosing levels may result in higher tracer accumulation without significantly increasing background fluorescence.^{25,26} This may further improve the detection of tumor-positive CRMs that are based on small tumor deposits, which proved to be challenging in one of our patients.

We propose a standardized imaging protocol that can be used in future research and for the development of FGL, based on our findings in the current study and our recently reported analytical workflow (Figure 5).²⁶ Fluorescence imaging is performed at fixed time-points. First, when feasible, intraoperative fluorescence-guided surgery is performed. After resection, back-table FGL of the fresh surgical specimen is performed to evaluate the resection margin status, preferably using a closed-field imaging system. In case high fluorescence is observed during intraoperative or back-table FGL, several treatment options can be considered, such as frozen section analysis, an additional or extended resection, IORT or potentially more innovative treatment modalities like photo immunotherapy.¹² Subsequently, to correlate fluorescence with histology, fluorescence imaging is performed during histopathological processing: of the fresh surgical specimen, formalin-fixed tissue slices and paraffin-embedded tissue sections. Additionally, fluorescence microscopy can evaluate local tracer accumulation. Altogether, this can give more insight in tissue biodistribution, important for tracer or drug development, or to evaluate potential off- and on-target effects of fluorescence tracers. We believe such a standardized imaging protocol is widely applicable for the validation of FGL in different tumor types and with different fluorescent tracers.

Figure 5. Proposed data collection and analysis design for resection margin evaluation, including intraoperative imaging to evaluate resection margins and identify potential lymph nodes and/or peritoneal metastasis, back-table imaging of the fresh surgical specimen to evaluate resection margins, and further fluorescence analysis of tissue slices, formalin-fixed paraffin-embedded (FFPE) tissue blocks and tissue sections for cross-reference and correlation of fluorescence with histology. T = tumor; HE = hematoxylin eosin; IHC = immunohistochemistry; FM = fluorescence microscopy.

CONCLUSION

Our study shows the potential of back-table FGI using the near-infrared fluorescent tracer bevacizumab-800CW targeting VEGFA for margin evaluation at the surgical theatre in patients with LARC. The technique itself proved to be safe, feasible and shows potential to guide perioperative clinical decision-making with a high sensitivity in patients with a threatened tumor-positive resection margin. A phase II study using a standardized imaging protocol is in development to confirm these results. Future studies will provide evidence if FGI will be beneficial for all patients, or will only be applied in selected cases to guide clinical decision-making.

DISCLOSURE

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REFERENCES

- 1 Sauer R, Becker H, Hohenberger W, et al. Preoperative versus Postoperative Chemoradiotherapy for Rectal Cancer. *New Engl J Medicine* 2004; 351: 1731–40.
- 2 Sauer R, Liersch T, Merkel S, et al. Preoperative Versus Postoperative Chemoradiotherapy for Locally Advanced Rectal Cancer: Results of the German CAO/ARO/AIO-94 Randomized Phase III Trial After a Median Follow-Up of 11 Years. *J Clin Oncol* 2012; 30: 1926–33.
- 3 Bosset J-F, Collette L, Calais G, et al. Chemotherapy with Preoperative Radiotherapy in Rectal Cancer. *New Engl J Medicine* 2006; 355: 1114–23.
- 4 Gérard J-P, Conroy T, Bonnetain F, et al. Preoperative Radiotherapy With or Without Concurrent Fluorouracil and Leucovorin in T3-4 Rectal Cancers: Results of FFCD 9203. *J Clin Oncol* 2006; 24: 4620–5.
- 5 van der Valk MJ, Hilling DE, Bastiaannet E, et al. Long-term outcomes of clinical complete responders after neoadjuvant treatment for rectal cancer in the International Watch & Wait Database (IWWDD): an international multicentre registry study. *Lancet* 2018; 391: 2537–45.
- 6 Nagtegaal ID, Quirke P. What Is the Role for the Circumferential Margin in the Modern Treatment of Rectal Cancer? *J Clin Oncol* 2008; 26: 303–12.
- 7 Simillis C, Baird DL, Kontovounisios C, et al. A Systematic Review to Assess Resection Margin Status After Abdominoperineal Excision and Pelvic Exenteration for Rectal Cancer. *Ann Surg* 2017; 265: 291–9.
- 8 Nagtegaal ID, Marijnen CA, Kranenburg E, van de Velde CJ, van Krieken HJ. Circumferential Margin Involvement Is Still an Important Predictor of Local Recurrence in Rectal Carcinoma. *Am J Surg Pathology* 2002; 26: 350–7.
- 9 Mercury Study Group. Diagnostic accuracy of preoperative magnetic resonance imaging in predicting curative resection of rectal cancer: prospective observational study. *Brmj* 2006; 333: 779.
- 10 Tanaka S, Martling A, Lindholm J, Holm T, Palmer G. Remaining cancer cells within the fibrosis after neo-adjuvant treatment for locally advanced rectal cancer. *European J Surg Oncol Ejsa* 2015; 41: 1204–9.
- 11 Khoury W, Abboud W, Hershkovitz D, Duek SD. Frozen section examination may facilitate reconstructive surgery for mid and low rectal cancer. *J Surg Oncol* 2014; 110: 997–1001.
- 12 Mitsunaga M, Ogawa M, Kosaka N, Rosenblum LT, Choyke PL, Kobayashi H. Cancer cell-selective in vivo near infrared photoimmuno-therapy targeting specific membrane molecules. *Nat Med* 2011; 17: 1685–91.
- 13 Krajnović M, Marković B, Knežević-Ušaj S, et al. Locally advanced rectal cancers with simultaneous occurrence of KRAS mutation and high VEGF expression show invasive characteristics. *Pathology - Res Pract* 2016; 212: 598–603.
- 14 Zlobec I, Vuong T, Compton C, et al. Combined analysis of VEGF and EGFR predicts complete tumour response in rectal cancer treated with preoperative radiotherapy. *Brit J Cancer* 2008; 98: 450–56.
- 15 Ferrara N, Gerber H-P, LeCouter J. The biology of VEGF and its receptors. *Nat Med* 2003; 9: 669–76.
- 16 ter Weele EJ, van Scheltinga A, Linsen MD, et al. Development, preclinical safety, formulation, and stability of clinical grade bevacizumab-800CW, a new near infrared fluorescent imaging agent for first in human use. *Eur J Pharm Biopharm* 2016; 104: 226–34.
- 17 Dobosz M, Ntziachristos V, Scheuer W, Strobel S. Multispectral Fluorescence Ultramicroscopy: Three-Dimensional Visualization and Automatic Quantification of Tumor Morphology, Drug Penetration, and Antiangiogenic Treatment Response. *Neoplasia* 2014; 16: 1–13.
- 18 Boogerd LS, Hoogstins CE, Schaap DP, et al. Safety and effectiveness of SGM-101, a fluorescent antibody targeting carcinoembryonic antigen, for intraoperative detection of colorectal cancer: a dose-escalation pilot study. *Lancet Gastroenterology Hepatology* 2018; 3: 181–91.
- 19 Harlaar NJ, Koller M, de Jongh SJ, et al. Molecular fluorescence-guided surgery of peritoneal carcinomatosis of colorectal origin: a single-centre feasibility study. *Lancet Gastroenterology Hepatology* 2016; 1: 283–90.
- 20 Tummers WS, Warram JM, van den Berg NS, et al. Recommendations for reporting on emerging optical imaging agents to promote clinical approval. *Theranostics*; 8: 5336–47.

- 21 Alberda WJ, Verhoef C, Nuyttens JJ, et al. Intraoperative Radiation Therapy Reduces Local Recurrence Rates in Patients With Microscopically Involved Circumferential Resection Margins After Resection of Locally Advanced Rectal Cancer. *Int J Radiat Oncol Biology Phys* 2014; 88: 1032–40.
- 22 Hyngstrom JR, Tzeng CD, Beddar S, et al. Intraoperative radiation therapy for locally advanced primary and recurrent colorectal cancer: Ten-year institutional experience. *J Surg Oncol* 2014; 109: 652–8.
- 23 Ferenschild F, Vermaas M, Verhoef C, et al. Total Pelvic Exenteration for Primary and Recurrent Malignancies. *World J Surg* 2009; 33: 1502–8.
- 24 Trakarnsanga A, Gonen M, Shia J, et al. What is the Significance of the Circumferential Margin in Locally Advanced Rectal Cancer After Neoadjuvant Chemoradiotherapy? *Ann Surg Oncol* 2013; 20: 1179–84.
- 25 Hartmans E, Tjalma J, Linssen MD, et al. Potential Red-Flag Identification of Colorectal Adenomas with Wide-Field Fluorescence Molecular Endoscopy. *Theranostics* 2018; 8: 1458–67.
- 26 Koller M, Qiu S-Q, Linssen MD, et al. Implementation and benchmarking of a novel analytical framework to clinically evaluate tumor-specific fluorescent tracers. *Nat Commun* 2018; 9: 3739.

SUPPLEMENTAL MATERIAL

Supplemental material and methods

Fluorescence grid analysis

Tumor locations were delineated on 4 µm HE stained tissue sections by a board-certified gastrointestinal pathologist, who was blinded for fluorescence imaging results. The histological delineation was merged with the high-resolution fluorescence images of the formalin-fixed tissue slices (Odyssey CLx), to enable a direct correlation of fluorescence with histology. Subsequently, a fluorescence grid analysis was performed, which was adapted from Gao et al.¹ A 3 x 3 mm grid was drawn on the merged image using ImageStudio software (version 5.0, LI-COR Biosciences Inc., Lincoln, NE, USA), dividing it into identical 9 mm² squares. Each square was classified as tumor-negative or tumor-positive if more than 20% of the square consisted of tumor-tissue, based on the histological delineation. ImageStudio software automatically calculated mean fluorescence intensities (MFI) for each square. A receiver operating characteristics (ROC) curve was determined per patient and for all patients combined, to determine an optimal cut-off value for tumor detection based on optimal sensitivity and specificity as determined by Youden's J statistics.

Three-dimensional tissue analysis by light-sheet fluorescence microscopy

LSFM enables a multicolor 3D analysis of optical transparent whole-mount tissue specimen at cellular resolution. Tissue slices were formalin fixated, dehydrated, and incubated in an organic clearing solution (one-part benzylalcohol & two-parts benzylbenzoate, incubation condition: 24-48 hours, 4°C, dark) to obtain high optical tissue transparency. Subsequently, the fluorescence signal intensity of bevacizumab-800CW as well as the tissue autofluorescence (providing detailed morphological tissue information) were measured within the cleared rectal cancer tissue using a commercially available light-sheet microscope (UltraMicroscope II, LaVision Biotec GmbH, Bielefeld, Germany). The obtained fluorescence imaging results were visualized as single and co-registered data sets, combining information of drug penetration/accumulation with morphological tissue context. In addition, the performed virtual 3D tissue analysis was also correlated to conventional histology.

Fluorescence imaging parameters

Throughout the study, the same imaging parameters were used for data acquisition per imaging device. In case of saturation of fluorescence images, the fluorescence exposure time or gain was decreased to ensure adequate data collection.

Supplemental reference

- 1 Gao RW, Teraphongphom NT, van den Berg NS, et al. Determination of tumor margins with surgical specimen mapping using near-infrared fluorescence. *Cancer Research* 2018; 78: 5144-5154.

Supplemental figures

Supplemental Figure 1. Flow diagram of the study. From 25 patients, eight patients were included in this explorative analysis for the use of back-table fluorescence-guided imaging to evaluate the circumferential resection margins (CRM) status on the fresh surgical specimens. Out of two tumor-positive margins, one was identified correctly using back-table, whereas the other was considered tumor-negative, despite the presence of (sub)millimeter tumor deposits <1 mm of the CRM. Five out of six tumor-negative CRMs were identified correctly. The remaining CRM was identified to be tumor-positive based, although the CRM was marginally negative CRM with 1.4 mm. Seventeen patients were included to determine the sensitivity and specificity of bevacizumab-800CW and evaluate local tracer accumulation, including the eight patients included for CRM evaluation.

Supplemental Figure 2. Tumor-positive CRM with low fluorescence intensities during back-table FGI of the fresh surgical specimen and fluorescence imaging of a subsequent tissue slice (A). The orange arrows indicate the location of the tumor-positive CRM that was based on isolated microscopic tumor deposits as determined on final histopathology (B). The asterisk () indicates a tumor-positive lymph node. FFPE = formalin-fixed paraffin embedded; HE = hematoxylin eosin.*

Supplemental Figure 3. Tumor-negative CRM with low fluorescence during back-table FGI of the fresh surgical specimen and a corresponding tissue slice, showing fluorescence at the location of the luminal tumor, though low fluorescence near the CRM (A), which was confirmed during microscopic fluorescence imaging and correlated to histology (B). FFPE = formalin-fixed paraffin embedded; HE = hematoxylin eosin.

Supplemental table

*Supplemental Table 1. Contingency table. A qualitative evaluation of fluorescence intensities was performed on the fresh surgical specimens. Low fluorescence was assessed as a homogenous background fluorescence; localized increased fluorescence was assessed as high fluorescence. *Distance to the circumferential resection margin (CRM) of 1.4 mm. **Tumor-positive CRM based on isolated microscopic tumor deposits ≤ 1 mm of the CRM.*

	Tumor-positive CRM	Tumor-negative CRM	Total
High fluorescence	1	1*	2
Low fluorescence	1**	5	6
Total	2	6	8

Supplemental video

Supplemental Video 1. Three-dimensional light-sheet microscopy of bevacizumab-800CW fluorescence localized in tumor environment of a tissue slice containing tumor. Tissue slice histology is also depicted in Figure 4. The left tissue slice depicts the tissue morphology based on autofluorescence; the right tissue slice depicts the near-infrared fluorescence image.

9

Summary, conclusions and future perspectives

SUMMARY AND CONCLUSIONS

Cancer of the digestive system is the most common form of cancer worldwide.¹ Early-stage and accurate cancer detection is of the utmost importance for survival. Despite improvements in high-definition white-light endoscopy (HD-WLE) and novel advanced endoscopic imaging techniques, there still is a significant miss-rate of (pre)malignant mucosal lesions.^{2–5} Fluorescence molecular endoscopy (FME) has the potential to improve mucosal lesion detection using targeted near-infrared (NIR) fluorescent imaging agents.

Although early detection of (pre)malignant lesions is crucial for prognosis, accurate tumor removal is equally important. The number of tumor-positive resection margins^{6–9} demonstrates visual inspection and palpation are often insufficient to discriminate between tumor and healthy tissue, as is frozen section analysis.^{10,11} Fluorescence-guided surgery (FGS) has the potential to evaluate the resection margin status *in real-time*, using targeted NIR optical imaging agents at the operating theatre. **Chapter 1** describes these clinical challenges and provides a general introduction and outline of this thesis.

The aim of this thesis is to improve (pre)malignant lesion detection during endoscopy (**part I**) and to improve intraoperative tumor detection and resection margin evaluation during surgery (**part II**), through clinical application of targeted NIR fluorescent imaging agents.

Part I of this thesis describes currently available endoscopic imaging enhancement techniques and two clinical applications of FME using EMI-137.

In **Chapter 2**, we outlined the mechanism of action and current clinical status of the available endoscopic imaging enhancement techniques. Wide-field imaging techniques such as chromoendoscopy, narrow-band imaging and autofluorescence imaging already play a role in endoscopy for the detection of neoplastic lesions in inflammatory bowel disease and BE patients, and for characterization of colorectal polyps respectively. Relatively new in the field of endoscopy are virtual chromoendoscopy techniques, such as i-Scan (Pentax), Flexible Spectral Imaging Color Enhancement (FiCE, Fujinon) and STORZ Professional Image Enhancement System (SPIES, Karl Storz), which provide contrast through post-processing software that adjust the reflected light wavelength ranges. Cross-sectional endoscopic imaging techniques provide morphological or histological information, aiming to assist in lesion characterization. Optical coherence tomography (OCT) and second-generation OCT techniques such as volumetric laser endomicroscopy or tethered capsule endomicroscopy facilitate three-dimensional tissue morphology through use of a NIR low-coherence laser. Confocal laser endomicroscopy and endocytoscopy further improve image resolution and facilitate virtual histology at a cellular and subcellular level *in vivo*, providing ‘optical tissue biopsies’. Despite promising *in vivo* results, these techniques should be further validated.

Also relatively new in the field of endoscopy is optical molecular imaging. Optical molecular imaging has the potential to serve as a ‘red-flag’ imaging technique by providing *real-time* visualization of biomarker expression levels that become upregulated during the course of a disease. **Chapter 3** describes the optimal dose-to-imaging window for FME

using EMI-137 for colorectal polyp detection. We demonstrated that FME using 0.13 mg/kg of EMI-137 appears to be safe and feasible within a one-to-three hour timeframe, as increased fluorescence was visualized and subsequently quantified in the polyps at each dose-to-imaging interval, without clinically significant differences between the time-cohorts. We validated these results by correlating fluorescence intensities to histology and c-Met expression levels *ex vivo*. Overall, this expands the clinical utility of EMI-137 for improved lesion detection during screening endoscopies in a population at average CRC risk.

The lack of guidelines and standards in the field of FME has led to significant variation in the analysis methods and outcome parameters of previous FME studies.^{12–17} In **Chapter 4**, we report the first results on FME using EMI-137 in BE patients, using not only qualitative measurements such as the *in vivo* FME results or semi-quantification on FME images, but also quantitative MDSFR/SFF spectroscopy values. In 15 patients, 16 out of 18 neoplastic lesions were correctly identified using FME related to histology and c-Met expression levels, although no additional lesions were identified. We concluded that c-Met targeted FME may not be the optimal combination for lesion identification during surveillance endoscopies in BE patients due to the modest contrast observed, variability in c-Met expression in the neoplastic lesions and physiological c-Met expression in stomach-type epithelium. In **Chapter 4** we also identified six important study outcome-parameters for early phase FME studies: the optimal administration route; optimal dose and safety; *in vivo* FME contrast; quantification of intrinsic fluorescence; *ex vivo* correlation of fluorescence, histopathology and target expression; and microscopic tracer distribution. The proposed outcome parameters can serve as a valuable guideline for designing and performing future early phase clinical FME studies, independent of which fluorescent tracer is investigated.

Part II of this thesis provides an overview of the principles of FGS (**Chapter 5**) and describes the clinical application of FGS for tumor detection and resection margin evaluation in patients with colorectal peritoneal carcinomatosis (PC), head and neck squamous cell carcinomas (HNSCC) and locally advanced rectal cancer (LARC).

Chapter 6 shows for the first time that the clinical application of FGS using bevacizumab-800CW is safe and feasible during cytoreductive surgery of colorectal PC. We demonstrated that bevacizumab-800CW accumulates in the microenvironment of VEGFA overexpressing peritoneal metastases on a macroscopic to microscopic level, by correlating fluorescence intensities with histology and VEGFA expression levels *ex vivo*. We conclude that this technique has the potential to improve cytoreduction and patient selection, as we demonstrated that in the absence of fluorescence, no residual disease was detected. These results have led to the design of a subsequent phase-II diagnostic accuracy study.

The quantitative dose-escalation study we describe in **Chapter 7** showed the safety, feasibility and optimal dose of FGS using cetuximab-800CW for HNSCC detection and resection margin evaluation. The optimal dose of cetuximab-800CW appeared to be 15 mg cetuximab-800CW preceded by a pre-dose of 75 mg unlabeled cetuximab. Out

of 15 patients, all four tumor-positive resection margins were correctly identified using back-table fluorescence-guided imaging (FGI) at the operating theater. Extensive *ex vivo* validation steps showed that the binding localization of cetuximab-800CW was around the tumor cell membranes on a microscopic level. Interestingly, we were able to use a two-to-threefold lower cetuximab-800CW dose compared to previous research¹⁸, while still obtaining sufficient tumor-to-background ratios for tumor detection and resection margin evaluation. This might reduce the risks for side-effects and toxicity. These results were confirmed by *in* and *ex vivo* quantification of intrinsic fluorescence values using MDSFR/SFF spectroscopy as an additional parameter to create more objective and quantitative fluorescence values.

The emphasis of FGI has mainly been on the intraoperative application to guide clinical decision making. However, back-table FGI of the fresh surgical specimen provides several advantages, as closed-field fluorescence imaging can be performed in a controlled environment, standardizing the camera angle, imaging distance and illumination homogeneity. In the proof-of-concept study described in **Chapter 8**, we demonstrated the safety and potential clinical value of back-table FGI using bevacizumab-800CW for LARC resection margin evaluation. We showed that fluorescence intensities correlated with histology on a macroscopic and microscopic level. To make FGI less of a qualitative assessment, we determined a fluorescence cut-off value that enabled tumor detection with a sensitivity and specificity of 96.16% and 80.39% respectively. We concluded back-table FGI might have the potential to improve resection margin status evaluation in LARC patients, although future studies are required to confirm these results in a larger cohort. Finally, we proposed a standardized methodology to assist in the future development of back-table FGI.

FUTURE PERSPECTIVES

Although optical molecular imaging using fluorescent imaging agents is still in its infancy, the potential benefits of the technique have been demonstrated by the early phase clinical studies described in this thesis. However, several advances are required to move the technique forward into standard clinical care.

First of all, technological improvements may further optimize fluorescence imaging. Two highly sensitive fluorescence cameras were used in the clinical studies described in this thesis, which were optimized for the endoscopic detection of EMI-137 (**Chapter 3** and **4**) and for the intraoperative detection of IRDye800CW (**Chapter 6, 7** and **8**). During endoscopy, a fiber-based approach was used for FME to enable HD-WLE in parallel to real-time fluorescence and white-light imaging.^{19,20} However, this approach does limit the imaging resolution and field-of-view, which could be improved by the development of an integrated fluorescence video-endoscope. The main challenge is to develop a near-infrared (NIR) CCD-chip that is sensitive enough for the visualization of nanomolar tracer concentrations. A second advantage of an integrated fluorescence video-endoscope, is that the working-channel remains available for interventions or targeted biopsies.

In parallel, wide-field FGS systems could be further improved by decreasing the size and flexibility of the camera head, or improving currently available laparoscopic or robotic fluorescence cameras, as these lack sensitivity to detect nanomolar tracer concentrations. Furthermore, multispectral imaging could be incorporated in fluorescence imaging devices, to enable separation of different spectra of light, which might improve imaging specificity and allow detection of multiple targeted optical imaging agents at separate wavelengths.

Furthermore, MDSFR/SFF spectroscopy was used to quantify intrinsic fluorescence intensities by correcting the observed fluorescence for the tissue optical properties (Chapter 3, 4 and 7). Although MDSFR/SFF spectroscopy only provides small pin-point measurements, we believe quantification of intrinsic fluorescence contributes in a complementary, quantitative manner to more objective decision making in dose-to-imaging interval (Chapter 3) or dose-finding (Chapter 4 and 7) studies and may provide insight in drug distribution. In the future, this might enable more accurate tumor discrimination by application of a quantitative threshold for tumor detection. However, MDSFR/SFF measurements are currently calculated in retrospect using a post-processing algorithm. In order to provide *real-time* objective intrinsic fluorescence values, we are further developing the technique in close collaboration with the Erasmus Medical Center, Rotterdam, The Netherlands.

Despite abovementioned technical improvements, optical molecular imaging is still subject to a limited penetration depth due to absorption of light by the tissue. A different technique that has the potential to further improve image resolution and imaging penetration depth, is multispectral optoacoustic tomography (MSOT). MSOT is a *real-time* imaging technique that has the potential to enable high resolution, two-or-three-dimensional, non-invasive imaging of human tissue at several centimeters of penetration depth, by combining near-infrared optoacoustic imaging and ultrasonography.^{21,22} Multiple wavelengths of short-pulsed NIR laser light are used to illuminate the tissue of interest. Photo-absorbing molecules, which can either be endogenous molecules (e.g. hemoglobin, melanin) or exogenous molecules (e.g. targeted imaging probes), absorb this laser light and subsequently undergo a thermoelastic expansion, which can be detected as ultrasound waves.^{21,22} Endogenous MSOT imaging has been evaluated in Crohn's disease patients, suggesting evaluation of hemoglobin levels using MSOT has the potential to discriminate between active disease and remission²³ and in patients with Duchenne muscular dystrophy (NCT03490214). The feasibility of exogenous MSOT imaging is currently under investigation using intravenous administration of bevacizumab-800CW and cetuximab-800CW for detection of atherosclerotic plaque instability and cervical lymph node metastases respectively (NCT03757507; NCT03923881).

In parallel to the optimization of fluorescence imaging devices, the search for new biomarkers and optical imaging agents continues, to improve imaging sensitivity and specificity. However, biomarker identification is challenging, as the downstream effects of genomic alterations that occur during the course of a disease may be overshadowed by non-genetic physiological or metabolic factors. A promising method that corrects gene expression data for non-genetic factors to provide insight in downstream effects on

receptor or protein level, is functional genomic mRNA (FMG) profiling.²⁴ FMG profiling has already been applied to identify targets for colorectal adenomas and pancreatic cancer detection, but can be used for other diseases as well.^{22,23,25,26} After biomarker identification, the Target Selection Criteria (TASC) scoring system can be applied to select potential biomarkers for development of tumor-targeted imaging agents, based on seven crucial target characteristics.^{25,27}

The development of optical imaging agents however is complex and translation into a cGMP compliant product and upscaling to larger quantities can be challenging. Several types of carrier molecules can be conjugated to a fluorescent dye for disease targeting, such as antibodies, peptides, small molecules or activatable probes.^{25,28} Recently, a roadmap has been designed for the development and clinical translation of antibody based optical imaging agents, which can guide the development of an investigational medicinal product that is suitable for human use.^{24,29} Peptides and small molecules generally have favorable pharmacokinetics due to their relatively small size, although as a result of that, conjugation to a fluorescent dye can be more challenging. Furthermore, activatable fluorophores can be cleaved by enzymes and therefore only emit fluorescence after quenching. This binary ‘on/off’ approach has for example been implemented in the development of the near-infrared fluorescent tracer ONM-100. ONM-100 is a pH-activatable micellar polymer compound loaded with quenched ICG, which is released and un-quenched upon dissociation in the presence of a lower pH.^{28,30} ONM-100 therefore does not rely on the binding to a specific biomarker for tumor targeting, but relies on cancer acidosis to become activated. As tumor acidosis is considered a hallmark of cancer, we believe this tracer shows great potential to serve as a generic tool for cancer detection. Recently, ONM-100 has been translated into the clinic for FGS of breast, esophageal, colorectal and head and neck cancer patients (Dutch Trial Register: NTR7085).

Only several indications for FME and FGS have been demonstrated in this thesis. However, there are numerous other indications that may benefit from optical molecular imaging. Firstly, FME may be used to evaluate treatment response or to gain insight in drug distribution levels. This was demonstrated for the first time in patients with Crohn’s disease, where fluorescently conjugated anti-tumor necrosis factor (TNF) antibodies were used as a read-out for biomarker expression levels, in order to predict treatment response.¹⁷ More recently, a similar approach was investigated using VEGFA-targeted quantitative fluorescence endoscopy to identify locally advanced rectal cancer patients¹⁴ or esophageal adenocarcinoma patients (NCT03558724) with a complete response after neoadjuvant chemoradiotherapy. Furthermore, optical molecular imaging may also be of added value for other endoscopic or laparoscopic imaging techniques, such as in pulmonology, urology or surgery, for improved tumor visualization or treatment response evaluation. Finally, FGI may also play a role for the pathologist. Gross pathology can be time-consuming, especially when the tissue architecture has changed due to for example chemoradiotherapy. We hypothesize FGI may be of added value in discriminating tumor from benign or fibrotic tissue, or for the lymph node identification, although this would need to be confirmed in a future study.

As optical molecular imaging is progressing as a technique, there is more and more emphasis on standardization in fluorescence imaging methodologies^{31–33}, which we have also described and optimized for FME in **Chapter 4**. However, the use of fluorescence imaging cameras with a different sensitivity, dynamic range and image processing software also highly influence FGI results. Consequently, we believe there may be an added value for incorporation of a multiparametric phantom in clinical studies, which enables evaluation of the dynamic range, sensitivity, illumination homogeneity and resolution of a fluorescence camera.^{26,27,34} This not only verifies adequate performance of the fluorescence camera per imaging procedure, it also allows comparison of different fluorescence cameras in clinical studies.

With so many potential indications, ultimately the volume and complexity of the fluorescence imaging data might be too much for human analysis. We believe that computer-aided detection (CAD) algorithms may increase the amount of information that can be processed and interpreted, using artificial intelligence or machine-learning techniques to improve the recognition of disease-related abnormal patterns in imaging data. The potential benefit of CAD has already been demonstrated for several endoscopic imaging techniques, to diagnose neoplastic BE-lesions using HD-WLE^{35,36} or volumetric laser endomicroscopy.^{35,37} Future studies would need to demonstrate the added value for improved interpretation of fluorescence imaging data.

The next step to move optical molecular imaging in endoscopy and surgery forward, is to validate initial phase-I study results in larger patient cohorts, focusing on the clinical benefit by evaluating the diagnostic accuracy in terms of sensitivity and specificity. For the clinical studies described in **Chapter 3** and **6**, subsequent studies are being designed, while a phase-II clinical study of **Chapter 7** has already been initiated at our institute (NCT03134846). To determine the added value of FME and FGS for future application, clinical decision making has to become adherent to fluorescence imaging results at some point as well.

In conclusion, this thesis describes the application of different fluorescent imaging agents for use in endoscopy and surgery. Future clinical phase-II studies should determine the potential clinical benefit for improved (pre)malignant lesion detection using FME, and for improved oncological surgery and evaluation of the resection margin status using FGS and back-table FGI.

REFERENCES

- Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *Ca Cancer J Clin* 2018; 68: 394–424.
- Vieth M, Ell C, Gossner L, May A, Stolte M. Histological Analysis of Endoscopic Resection Specimens From 326 Patients with Barrett's Esophagus and Early Neoplasia. *Endoscopy* 2004; 36: 776–81.
- Shaheen NJ, Falk GW, Iyer PG, Gerson LB. ACG Clinical Guideline: Diagnosis and Management of Barrett's Esophagus. *Am J Gastroenterology* 2016; 111: 30.
- Atkin W, Wooldrage K, Parkin DM, et al. Long term effects of once-only flexible sigmoidoscopy screening after 17 years of follow-up: the UK Flexible Sigmoidoscopy Screening randomised controlled trial. *Lancet* 2017; 389: 1299–311.
- Rijn JC van, Reitsma JB, Stoker J, Bossuyt PM, Deventer SJ van, Dekker E. Polyp Miss Rate Determined by Tandem Colonoscopy: A Systematic Review. *Am J Gastroenterol* 2006; 101: 343–50.
- Woolgar JA, Triantafyllou A. A histopathological appraisal of surgical margins in oral and oropharyngeal cancer resection specimens. *Oral Oncol* 2005; 41: 1034–43.
- Smits RWH, Koljenović S, Hardillo JA, et al. Resection margins in oral cancer surgery: Room for improvement. *Head Neck* 2016; 38: E2197–203.
- Simillis C, Baird DLH, Kontovounisios C, et al. A Systematic Review to Assess Resection Margin Status After Abdominoperineal Excision and Pelvic Exenteration for Rectal Cancer. *Ann Surg* 2017; 265: 291–9.
- MERCURY Study Group. Diagnostic accuracy of preoperative magnetic resonance imaging in predicting curative resection of rectal cancer: prospective observational study. *Bmj* 2006; 333: 779.
- DiNardo LJ, Lin J, Karageorge LS, Powers CN. Accuracy, Utility, and Cost of Frozen Section Margins in Head and Neck Cancer Surgery. *Laryngoscope* 2000; 110: 1773–6.
- Khoury W, Abboud W, Hershkovitz D, Duek SD. Frozen section examination may facilitate reconstructive surgery for mid and low rectal cancer. *J Surg Oncol* 2014; 110: 997–1001.
- Burggraaf J, Kamerling IMC, Gordon PB, et al. Detection of colorectal polyps in humans using an intravenously administered fluorescent peptide targeted against c-Met. *Nat Med* 2015; 21: 955–61.
- Nagengast WB, Hartmans E, Garcia-Allende PB, et al. Near-infrared fluorescence molecular endoscopy detects dysplastic oesophageal lesions using topical and systemic tracer of vascular endothelial growth factor A. *Gut* 2019; 68: 7–10.
- Tjalma JJ, Koller M, Linssen MD, et al. Quantitative fluorescence endoscopy: an innovative endoscopy approach to evaluate neoadjuvant treatment response in locally advanced rectal cancer. *Gut* 2019; : 1-5 Epub ahead of print.
- Hartmans E, Tjalma JJJ, Linssen MD, et al. Potential Red-Flag Identification of Colorectal Adenomas with Wide-Field Fluorescence Molecular Endoscopy. *Theranostics* 2018; 8: 1458–67.
- Joshi BP, Dai Z, Gao Z, et al. Detection of Sessile Serrated Adenomas in the Proximal Colon Using Wide-Field Fluorescence Endoscopy. *Gastroenterology* 2017; 152: 1002–13.
- Atreya R, Neumann H, Neufert C, et al. In vivo imaging using fluorescent antibodies to tumor necrosis factor predicts therapeutic response in Crohn's disease. *Nat Med* 2014; 20: nm.3462.
- Rosenthal EL, Warram JM, Boer E de, et al. Safety and Tumor Specificity of Cetuximab-IRDye800 for Surgical Navigation in Head and Neck Cancer. *Clin Cancer Res* 2015; 21: 3658–66.
- Garcia-Allende PB, Glatz J, Koch M, et al. Towards clinically translatable NIR fluorescence molecular guidance for colonoscopy. *Biomed Opt Express* 2013; 5: 78–92.
- Themelis G, Yoo JS, Soh K-S, Schulz R, Ntziachristos V. Real-time intraoperative fluorescence imaging system using light-absorption correction. *J Biomed Opt* 2009; 14: 064012-064012-9.
- Taruttis A, Ntziachristos V. Advances in real-time multispectral optoacoustic imaging and its applications. *Nat Photonics* 2015; 9: 219–27.
- Neuschmelting V, Burton NC, Lockau H, et al. Performance of a Multispectral Optoacoustic Tomography (MSOT) System equipped with 2D vs. 3D Handheld Probes for Potential Clinical Translation. *Photoacoustics* 2016; 4: 1–10.

- 23 Knieling F, Neufert C, Hartmann A, et al. Multispectral Optoacoustic Tomography for Assessment of Crohn's Disease Activity. *New Engl J Medicine* 2017; 376: 1292–4.
- 24 Fehrmann RSN, Karjalainen JM, Krajewska M, et al. Gene expression analysis identifies global gene dosage sensitivity in cancer. *Nat Genet* 2015; 47: ng.3173.
- 25 Koller M, Hartmans E, Groot DJA de, et al. Data-Driven Prioritization and Review of Targets for Molecular-Based Theranostic Approaches in Pancreatic Cancer. *J Nucl Med* 2017; 58: 1899–903.
- 26 Hartmans E, Orian-Rousseau V, Matzke-Ogi A, et al. Functional Genomic mRNA Profiling of Colorectal Adenomas: Identification and in vivo Validation of CD44 and Splice Variant CD44v6 as Molecular Imaging Targets. *Theranostics* 2017; 7: 482–92.
- 27 Oosten M van, Crane LMA, Bart J, Leeuwen FW van, Dam GM van. Selecting Potential Targetable Biomarkers for Imaging Purposes in Colorectal Cancer Using TArget Selection Criteria (TASC): A Novel Target Identification Tool. *Transl Oncol* 2011; 4: 71–82.
- 28 Zhang RR, Schroeder AB, Grudzinski JJ, et al. Beyond the margins: real-time detection of cancer using targeted fluorophores. *Nat Rev Clin Oncol* 2017; 14: 347.
- 29 Linssen MD, Weele EJ ter, Allersma DP, et al. Roadmap for the Development and Clinical Translation of Optical Tracers Cetuximab-800CW and Trastuzumab-800CW. *J Nucl Med* 2019; 60: 418–23.
- 30 Zhao T, Huang G, Li Y, et al. A transistor-like pH nanoprobe for tumour detection and image-guided surgery. *Nat Biomed Eng* 2016; 1: 0006.
- 31 Koch M, Symvoulidis P, Ntziachristos V. Tackling standardization in fluorescence molecular imaging. *Nat Photonics* 2018; 12: 505–15.
- 32 Koller M, Qiu S-Q, Linssen MD, et al. Implementation and benchmarking of a novel analytical framework to clinically evaluate tumor-specific fluorescent tracers. *Nat Commun* 2018; 9: 3739.
- 33 Hoogstins C, Burggraaf JJ, Koller M, et al. Setting Standards for Reporting and Quantification in Fluorescence-Guided Surgery. *Mol Imaging Biol* 2019; 21: 11–8.
- 34 Gorpas D, Koch M, Anastasopoulou M, et al. Multi-parametric Standardization of Fluorescence Imaging Systems Based on a Composite Phantom. *Ieee T Bio-med Eng* 2019; PP: 1–1.
- 35 Swager A-F, Sommen F van der, Klomp SR, et al. Computer-aided detection of early Barrett's neoplasia using volumetric laser endomicroscopy. *Gastrointest Endosc* 2017; 86: 839–46.
- 36 Shin D, Lee MH, Polydorides AD, et al. Quantitative analysis of high-resolution microendoscopic images for diagnosis of neoplasia in patients with Barrett's esophagus. *Gastrointest Endosc* 2016; 83: 107–14.
- 37 Ughi GJ, Gora MJ, Swager A-F, et al. Automated segmentation and characterization of esophageal wall in vivo by tethered capsule optical coherence tomography endomicroscopy. *Biomed Opt Express* 2016; 7: 409–19.

Appendix

Nederlandse samenvatting (*Dutch summary*)

Curriculum Vitae

Publicatielijst (*List of publications*)

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NEDERLANDSE SAMENVATTING (DUTCH SUMMARY)

Kanker is één van de meest voorkomende doodsoorzaken wereldwijd en met 18.1 miljoen nieuwe gevallen (2018) stijgt de incidentie nog altijd. De meest voorkomende vorm is kanker van het spijsverteringskanaal. Dit proefschrift richt zich op de (vroeg) detectie en behandeling van drie solide tumoren van het spijsverteringskanaal en hun voorstadia: het mondholte-carcinoom, het slokdarmcarcinoom en het colorectaal carcinoom.

Barrett oesofagus (BE) is een voorstadium van slokdarmkanker die wordt veroorzaakt door gastro-oesofageale reflux, waardoor het plaveiselcel epitheel wordt vervangen door cilindrisch epitheel met intestinale metaplasie. Maligne degeneratie van het BE-epitheel is een stapsgewijs proces, waarbij de intestinale metaplasie zich via laag- en hooggradige dysplasie ontwikkelt tot een carcinoom. Het gros van de colorectale carcinomen ontstaat via hetzelfde stapsgewijze proces, maar komt voort uit adenomateuze of sessiele serrated poliepen.

High-definition wit-licht endoscopie (HD-WLE) is de gouden standaard voor de detectie en behandeling van dergelijke premaligne mucosale laesies. Echter zijn de laesies vaak klein en de morfologische mucosale afwijkingen vaak subtiel. Mede hierdoor wordt een aanzienlijk deel van deze laesies gemist, terwijl de endoscopische behandel mogelijkheden voor dit soort laesies momenteel toenemen dankzij de ontwikkeling van geavanceerde technieken zoals endoscopische mucosale resecties (EMR), submucosale dissecties of full-thickness resecties. Dit maakt vroege laesie detectie dus extra belangrijk.

Als endoscopische behandeling niet mogelijk is, wordt er vaak een chirurgische resectie uitgevoerd. Tumor-vrije resectiemarges verbeteren de prognose en overleving significant en verminderen het risico op een lokaal recidief. Momenteel maakt de chirurg intra-operatief onderscheid tussen tumor en gezond weefsel door visuele inspectie en palpatie. Bij twijfel over de status van een resectiemarge kan een vriescoupe genomen worden, hoewel dit tijdrovend is en een risico geeft op sampling error. Het aanzienlijke aantal tumor-positieve resectiemarges anno 2019 toont aan dat de huidige technieken niet toereikend zijn.

Derhalve is er een evidente noodzaak voor een nieuwe techniek die de endoscopische detectie van (pre)maligne afwijkingen kan verbeteren, alsmede de intra-operatieve kanker detectie. Optische moleculaire beeldvorming is een opkomende techniek die contrast genereert door gebruik van nabij-infrarode (NIR) fluorescente tracers, die specifiek binden aan bepaalde bio-markers die door ziekte verhoogd tot expressie worden gebracht. Het principe van fluorescentie beeldvorming is gebaseerd op de excitatie van een fluorophore met een bepaalde golflengte, die daarna licht uitzendt met een langere golflengte. Deze fluorescentie kan worden gedetecteerd met een camerasysteem. Fluorescentie wordt beïnvloed door verschillende factoren, waaronder de optische eigenschappen van het weefsel (scattering en absorptie). Het NIR-lichtspectrum (650-900nm) biedt gunstige eigenschappen voor fluorescentie beeldvorming, omdat er relatief weinig weefselabsorptie en autofluorescentie is, waardoor de penetratiediepte toeneemt tot ± 1 cm. Fluorescentie beeldvorming kan gebruikt worden als 'red-flag' beeldvormingstechniek, om endoscopisch of intra-operatief beter onderscheid te kunnen maken tussen (pre)maligne en benigne weefsel.

De voornaamste **doelstellingen** van dit proefschrift zijn het verbeteren van de endoscopische detectie van (pre)maligne laesies en van de real-time, intra-operatieve tumor detectie, door gebruik van fluorescentie beeldvorming middels doelgerichte, NIR fluorescente tracers.

Deel I van dit proefschrift omschrijft de klinische toepassing van fluorescentie moleculaire endoscopie (FME). Om allereerst de endoscopische mogelijkheden voor mucosale laesie detectie in kaart te brengen, beschrijven wij in **Hoofdstuk 2** het werkingsmechanisme en de klinische status van de huidige endoscopische beeldvormingstechnieken. Deze worden onderverdeeld in ‘wide-field’ technieken voor macroscopische laesie detectie, en ‘cross-sectional’ technieken voor de visualisatie van microscopische details, zoals virtuele histologie.

FME is een voorbeeld van een wide-field beeldvormingstechniek. In het verleden is aangetoond dat FME met EMI-137, een fluorescent peptide gericht op de membraanreceptor c-Met, de potentie heeft om de colorectale poliep detectie miss-rate van 22% te verbeteren. Echter, het destijds onderzochte tijdsinterval tussen EMI-137 toediening en endoscopie van drie uren beperkt de klinische toepasbaarheid. In **Hoofdstuk 3** tonen wij aan dat er geen klinisch significant verschil bleek te zijn tussen een tijdsinterval van één tot drie uren voor colorectaal adenoom detectie middels FME. Deze resultaten zijn onderbouwd door *in vivo* kwantificatie van intrinsieke fluorescentie signalen middels multi-diameter, single-fiber reflectie, single-fiber fluorescentie (MDSFR/SFF) spectroscopie en *ex vivo* analyses. MDSFR/SFF-spectroscopie is een techniek die de fluorescentie intensiteit van de tracer corrigeert voor de optische eigenschappen van het weefsel zelf, zoals scattering en absorptie.

De afgelopen jaren zijn er meerdere fase-I FME studies uitgevoerd. Het ontbreekt echter nog aan standaarden die de doelstelling, uitkomstmaten en beeldvorming specificaties beschrijven. In **Hoofdstuk 4** beschrijven wij de eerste resultaten van een klinische studie naar FME met EMI-137 in BE-patiënten, waarbij we gebruik maken van niet alleen kwalitatieve uitkomstmaten zoals de FME-resultaten of semi-kwantificatie op FME-afbeeldingen, maar ook kwantitatieve uitkomstmaten zoals de MDSFR/SFF-spectroscopie resultaten. FME bleek haalbaar en veilig met verschillende doseringen EMI-137, na zowel intraveneuze als topicale toediening. In totaal werden 16 van de 18 (pre)maligne laesies gedetecteerd met FME, hoewel met gematigd contrast. De c-Met expressie-niveaus van de laesies varieerde, terwijl er fysiologische c-Met expressie werd geobserveerd in het maagtype epitheel. Concluderend bleek c-Met gerichte FME met EMI-137 niet de meest ideale combinatie voor het verbeteren van de (pre)maligne laesie detectie in BE-patiënten. In **Hoofdstuk 4** beschrijven we daarnaast zes belangrijke uitkomstmaten, die van belang zijn voor het opzetten en uitvoeren van klinische fase-I FME studies, te weten het onderzoeken van de optimale manier van toediening, dosering en veiligheid van een fluorescente tracer, maar ook de potentiële klinische toegevoegde waarde gebaseerd op *in vivo* FME en kwantificatie van fluorescentie door correctie voor optische eigenschappen. Daarnaast dient de specificiteit van de fluorescente tracer gevalideerd te worden door de fluorescentie te correleren aan de histologie en bio-marker/target expressieniveaus. De

voorgestelde uitkomstmaten kunnen als waardevolle toevoeging dienen om toekomstige fase-1 klinische studies richting te geven ongeacht van welke fluorescente tracer wordt onderzocht.

Deel II van dit proefschrift omschrijft de klinische toepassing van fluorescentie-geleide chirurgie (FGS) en pathologie. In **Hoofdstuk 5** beschrijven wij de principes van FGS, alsmede een chronologisch overzicht van de ontwikkeling van FGS toegespitst op peritoneale metastasen van het colorectaal carcinoom.

Peritoneale metastasen zijn metastasen op het buikvlies of de organen in de buikholte, die in opzet curatief behandeld kunnen worden middels cytoreductieve chirurgie gecombineerd met verwarmde intraperitoneale chemotherapie. Voor het succes van deze operatie is het cruciaal dat alle macroscopisch zichtbare metastasen worden geresecteerd. **Hoofdstuk 6** toont de resultaten van de eerste klinische fase-I studie, waarin FGS wordt gebruikt om colorectale peritoneale metastasen beter zichtbaar te maken met bevacizumab-800CW, een NIR fluorescente tracer gericht op de vasculaire endotheliale groeifactor-A (VEGF-A). Niet alleen was FGS met bevacizumab veilig en haalbaar, het bleek ook dat in de afwezigheid van fluorescentie, er geen tumorcellen aanwezig waren. Daarnaast toonden wij aan dat bevacizumab-800CW accumuleerde in de micro-omgeving van de peritoneale metastasen die VEGFA tot expressie brachten. Ondanks de kleine aantallen in deze studie, toont dit de potentie van FGS voor verbeterde cytoreductieve chirurgie en patiënt selectie. Dit heeft ertoe geleid dat er een fase-II studie naar de diagnostische nauwkeurigheid van deze techniek is opgezet in een soortgelijke patiëntenpopulatie.

In **Hoofdstuk 7** beschrijven wij een klinische studie die de veiligheid, haalbaarheid en optimale dosering onderzoekt voor FGS met cetuximab-800CW, een NIR fluorescente tracer gericht op de epidermale groeifactor receptor (EGFR), voor de verbeterde tumordetectie en resectiemarge evaluatie in patiënten met een mondholtecarcinoom. Momenteel heeft 20-40% van deze patiënten een tumor-positief snijvlak na tumorresectie. In de huidige studie werden uit 15 patiënten, alle vier de tumor-positieve resectiemarges terecht geïdentificeerd middels 'back-table' fluorescentie beeldvorming op de operatiekamer. De optimale dosering blijkt 75mg ongeconjugeerd cetuximab-800CW te zijn, gevolgd door 15mg cetuximab-800CW. In tegenstelling tot een eerdere klinische studie konden wij een twee-tot-driemaal lagere dosering toedienen zonder afbreuk te doen aan de sensitiviteit voor tumordetectie, door gebruik van een geoptimaliseerd camerasysteem. Deze resultaten werden bevestigd door *in vivo* kwantificatie van fluorescentie met MDSFR/SFF-spectroscopie. Momenteel wordt er een fase-II studie uitgevoerd in het UMCG om deze resultaten te valideren in een cohort van 70 patiënten (NCT03134846).

Tenslotte hebben wij de veiligheid en potentieel klinisch toegevoegde waarde aangetoond van back-table fluorescentie beeldvorming met bevacizumab-800CW in een proof-of-concept studie naar het evalueren van de resectiemarges bij patiënten met een lokaal uitgebreid rectumcarcinoom (**Hoofdstuk 8**). Na tumorresectie heeft 18.6% van deze patiënten een tumor-positieve resectiemarge, waarna de behandelopties beperkt zijn. Intra-operatieve vriescouples kunnen uitgevoerd worden om de status van het resectievlak

te beoordelen, maar deze zijn tijdrovend en hebben een grote kans op 'sampling error'. De huidige gouden standaard voor het evalueren van resectiemarges is histopathologie, wat tot zeven dagen kan duren. Back-table fluorescentie beeldvorming heeft de potentie om de resectiemarges binnen 10-15 minuten te evalueren op de operatiekamer, aangezien wij hebben aangetoond dat de gedetecteerde fluorescentie signalen correleerden met histologie. Daarnaast hebben wij een nieuwe methode door ontwikkelt om de interpretatie van fluorescentie signalen objectiever te maken, die ook gebruikt kan worden in andere toekomstige studies.

De conclusie en discussie over de bevindingen van dit proefschrift staan beschreven in **Hoofdstuk 9**, alsmede de toekomstperspectieven van fluorescentie-geleide endoscopie en chirurgie.

CURRICULUM VITAE

Steven Jakob de Jongh werd geboren op 5 december 1990 te Groningen. In 2008 behaalde hij zijn gymnasiumdiploma aan het Praedinius Gymnasium in Groningen. Na een jaar farmacie te hebben gestudeerd aan de Rijksuniversiteit Groningen, begon hij in 2009 met de studie geneeskunde aan de Rijksuniversiteit Groningen.

Steven volgde zijn coschappen in het Universitair Medisch Centrum Groningen (UMCG) en het Sint Elisabeth Hospitaal in Willemstad, Curaçao. Vervolgens deed hij zijn stage wetenschap op de afdeling oncologische chirurgie van het UMCG, bij de Optical Molecular Imaging Group onder supervisie van prof. dr. G.M. van Dam. Na zijn semi-arts stage op de afdeling chirurgie en plastische chirurgie van het Martini Ziekenhuis te Groningen, rondde hij zijn studie geneeskunde in april 2016 af. Tijdens zijn studie was Steven actief in diverse studie-gerelateerde commissies. Tevens heeft hij een studententeam opgezet op de afdeling chirurgie (ONKOS-studententeam), dat onder andere een jaarlijks symposium organiseert.

Na zijn afstuderen begon Steven met zijn promotieonderzoek in het UMCG onder supervisie van prof. dr. R.K. Weersma, prof. dr. G.M. van Dam en dr. W.B. Nagengast, wat heeft geresulteerd tot dit proefschrift. Tijdens zijn promotietraject heeft hij meerdere (internationale) congressen bezocht om zijn onderzoeksresultaten te presenteren, met als hoogtepunt een plenaire voordracht op de SPIE Photonics West in San Fransisco (VS), een prijs voor beste voordracht op de AIO-meeting van het Cancer Research Center en een prijs voor beste poster presentatie op de European Molecular Imaging Meeting in Glasgow (Schotland). Daarnaast heeft hij meerdere studenten begeleid met hun stage wetenschap. In de afrondende fase van zijn promotietraject initieerde Steven een nieuwe klinische studie op de afdeling plastische chirurgie, om het onderzoek uit zijn promotietraject een vervolg te geven.

Na de afronding van zijn promotietraject heeft Steven gewerkt als arts-assistent niet in opleiding tot specialist (ANIOS) Heelkunde op de afdeling chirurgie van het Martini Ziekenhuis Groningen. Momenteel is hij werkzaam als ANIOS plastische chirurgie in het Slingeland Ziekenhuis te Doetinchem. Steven woont samen met zijn vriendin Sanne Vreugdenhil in Groningen.

PUBLICATIELIJST (LIST OF PUBLICATIONS)

Voskuil FJ, **de Jongh SJ**, S.A.H.J. de Visscher, K.P. Schepman, W.B. Nagengast, et al. Fluorescentie geleide beeldvorming voor snijvlak evaluatie in hoofd-hals kankerpatiënten gebruikmakend van cetuximab-800CW: een kwantitatieve dosis escalatie studie.

Nederlands Tijdschrift voor Oncologie, 2020

Voskuil FJ*, Steinkamp PJ*, van der Vegt B, Dolff JJ, Jayalakshmi Y, et al, and the SHINE study group (o.a. **de Jongh SJ**). Exploiting metabolic acidosis in solid cancers using a tumo-agnostic pH-activatable nanoprobe for fluorescence-guided surgery.

Nature Communications, 2020

De Jongh SJ*, Vrouwe JPM*, Voskuil FJ, Schmidt I, Westerhof J, et al. The optimal imaging window for dysplastic colorectal polyp detection using c-Met targeted fluorescence molecular endoscopy.

Journal of Nuclear Medicine, 2020

De Jongh SJ*, Voskuil FJ*, Linssen MD, Hooghiemstra WTR, Linssen MD, et al. Fluorescence-guided imaging for resection margin evaluation in head and neck cancer patients using cetuximab-800CW: A quantitative dose-escalation study.

Theranostics, 2020

De Jongh SJ*, Voskuil FJ*, Schmidt I, Karrenbeld A, Kats-Ugurlu G, et al. C-Met targeted fluorescence molecular endoscopy in Barrett's esophagus patients and identification of outcome parameters for phase-I studies.

Theranostics, 2020

De Jongh SJ*, Tjalma JJJ*, Koller M, Linssen MD, Vonk J, et al. Back-table fluorescence-guided imaging for circumferential resection margin evaluation in locally advanced rectal cancer patients using bevacizumab-800CW.

Journal of Nuclear Medicine, 2019

Tjalma JJJ*, Koller M*, Linssen MD, Hartmans E, **de Jongh SJ**, et al. Quantitative fluorescence endoscopy: an innovative endoscopy approach to evaluate neoadjuvant treatment response in locally advanced rectal cancer.

Gut, 2019

De Jongh SJ*, Borgerink MMH*, Nagengast WB. A review on endoscopic imaging enhancement techniques.

Encyclopedia of Gastroenterology 2nd edition, 2019

De Jongh SJ*, Hentzen JEK*, Hemmer PHJ, van der Plas WY, van Dam GM, et al. Molecular fluorescence-guided surgery of peritoneal carcinomatosis of colorectal origin: a narrative review.

Journal of Surgical Oncology, 2018

Qiu SQ, Dorrius MD, **de Jongh SJ**, Jansen L, de Vries J, et al. Micro-computed tomography (micro-CT) for intraoperative surgical margin assessment of breast cancer: a feasibility study in breast conserving surgery.

European Journal of Surgical Oncology, 2018

Harlaar NJ*, Koller M*, **de Jongh SJ**, van Leeuwen BL, Hemmer PHJ, et al. Molecular fluorescence-guided surgery of peritoneal carcinomatosis of colorectal origin: a single-centre feasibility study.

Lancet Gastroenterology & Hepatology, 2016

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Steven de Jongh