Detection of esophageal Neoplasia by Wide-Field Fluorescence Molecular Endoscopy

Submitted, 2017

Elmire Hartmans,* Wouter Nagengast,* Pilar Garcia-Allende, Frans Peters, Matthijs Linssen, Maximilian Koch, Marjory Koller, Jolien Tjalma, Arend Karrenbeld, Annelies Jorritsma-Smit, Jan Kleibeuker, Gootitzen van Dam, Vasilis Ntziachristos

*Authors share co-first authorship

Department of Gastroenterology and Hepatology, Clinical Pharmacy and Pharmacology, Surgery, Pathology, Nuclear Medicine and Molecular Imaging and Intensive Care, University of Groningen, University Medical Center Groningen, Groningen, the Netherlands; Biological Imaging & Institute for Biological and Medical Imaging, Technical University of Munich and Helmholtz Center Munich, Munich, Germany
ABSTRACT

Background  Barrett’s esophagus (BE) undergo surveillance using high-definition (HD) white-light (WL) endoscopy with random four-quadrant biopsies. Nevertheless, the current random biopsy approach has an inherently high chance of sampling error, resulting in a high remaining dysplasia detection miss-rate of up to 57%. In a search for improving detection of esophageal dysplasia, we examined Fluorescence Molecular Endoscopy (FME) in a pilot study in BE patients, using systemic or topical administration of near-infrared (NIR) fluorescence-labeled bevacizumab, targeting vascular endothelial growth factor A (VEGFA).

Methods  In a prospective study, 14 BE patients underwent wide-field NIR-FME and white-light (WL) endoscopy, followed by an endoscopic mucosal resection (EMR). A microdose of the NIR-fluorescence labeled antibody was intravenously administered to five patients, two days prior to the procedure. The other nine patients received topical administration of the same agent, immediately before NIR-FME inspection.

Results  A total of 20 neoplastic lesions were identified during NIR-FME, of which four were not detected by WL endoscopy: 1 esophageal adenocarcinoma (EAC) and 3 histologically confirmed dysplastic areas. The additional lesions were all identified after the topical administration of the tracer. Overall, the topical administration resulted in higher in vivo tumor-to-background-ratios than the systemic approach (4.30 vs. 2.75). Ex vivo fluorescence-signal analyses confirmed increased uptake of the tracer in EAC and dysplasia compared to nondysplastic mucosa.

Conclusion  These proof-of-principle results demonstrate that NIR-FME can serve as a ‘red-flag’ aid to enhance diagnostic accuracy of WL endoscopy in BE patients, and provide justification for further clinical investigation.  ClinicalTrials.gov ID NCT02129933
INTRODUCTION

Worldwide, more than 450,000 persons are diagnosed with esophageal adenocarcinoma (EAC) per year, and approximately 400,000 die from the disease.\textsuperscript{1,2} Esophageal cancer is the eighth most commonly diagnosed cancer, but it is the sixth leading cause of cancer-related death, with incidence rates steeply rising.\textsuperscript{3,4} Although there are several risk factors for the development of EAC, including gastro esophageal reflux disease and obesity, intestinal metaplasia (IM) - also commonly known as Barrett’s esophagus (BE) - is only proven EAC precursor.\textsuperscript{5}

Malignant degeneration is a multistep process, in which IM cells can progress to low-grade (LGD), then to high-grade dysplasia (HGD), and finally to invasive cancer.\textsuperscript{6-8} However, endoscopic identification of early lesions is difficult, since macroscopic mucosal differentiation of LGD is challenging. Consequently, flat aberrant lesions harboring HGD and/or EAC are often missed.\textsuperscript{9,10} To remedy the detection problem, BE patients undergo surveillance endoscopy at designated intervals; generally every 3-5 years, using high-definition (HD) white-light (WL) endoscopy with random four-quadrant biopsies, taken every 1-2 cm along the affected BE segment.\textsuperscript{11} Nevertheless, the current random biopsy approach has an inherently high chance of sampling error, resulting in a high remaining detection miss-rate of up to 57%.\textsuperscript{12,13}

The requirement for early lesion identification has led to a search for new endoscopic techniques. Fluorescence imaging has been heralded as a promising method for detecting esophageal dysplasia and cancer,\textsuperscript{14} in particular when combined with agents with specificity to certain pathophysiological or biochemical parameters of the disease. In contrast to conventional endoscopic imaging techniques that detect disease on intrinsic contrast and superficial features, fluorescence imaging using targeted agents can enable surface and subsurface visualization going beyond the morphological features and tissue discolorations offered by HD-WL endoscopy. By targeting and ‘highlighting’ premalignant and malignant cells, fluorescence differentiation between normal and aberrant tissue could offer a method to better identify suspicious areas during endoscopic examination.\textsuperscript{15-19}

Confocal laser endomicroscopy (CLE) in combination with targeted agents has been considered for the detection of esophageal dysplasia\textsuperscript{20,21} This endoscopic approach can only sample small tissue areas (virtual biopsy) and it is not suitable for surveillance of the entire BE segment. Wide-field fluorescence imaging using an ASYNYDA-peptide labeled with the FITC-fluorophore has been explored for inspection of large esophagus segments in BE patients.\textsuperscript{21} However, concurrent operation of white light and fluorescence endoscopy in the visible spectrum limits the sensitivity and contrast available to the fluorescence method. Separation of weak fluorescence signals in the presence of strong white-light illumination
requires several orders of magnitude spectral separation through filters, which significantly reduces the collection sensitivity of fluorescence signals. Moreover, the visible exhibits strong auto-fluorescence, reducing imaging contrast. Therefore, the previous report of FME application required data post-processing for signal enhancement and did not allow real-time identification of lesions.

In this work we investigated for the first time real-time wide-field fluorescence molecular endoscopy (FME) in BE patients during the standard HD-WL endoscopic procedure, by switching fluorescence imaging to the near-infrared (NIR) spectral region. Separation of NIR fluorescence from white-light illumination can be very well accomplished with filters that do not significantly reduce fluorescence transmission. In addition, NIR fluorescence exhibits weaker auto-fluorescence than the visible, allowing for better contrast to background ratios. We examined the performance of vascular endothelial growth factor alpha (VEGFA) targeting tracer bevacizumab-800CW for esophageal adenocarcinoma and dysplasia detection in a pilot study of fourteen BE patients. Highly sensitive fluorescence sensing was achieved by developing a novel NIR-FME system that can endoscopically detect bevacizumab-800CW in real-time under microdosing administration conditions. We further compared for the first time the merits of systemic vs. topical administration of a fluorescence agent in a pilot patient population for real-time lesion detection during routine clinical inspection of the esophagus. We found that NIR-FME not only enabled previously undisclosed real-time wide-field NIR-FME esophageal surveillance but also detected after topical administration of the tracer remarkably more dysplastic and cancerous lesions, critically outperforming single white-light HD endoscopy.

RESULTS

In vivo imaging results
Imaging and biopsy protocol
Fourteen BE patients scheduled to undergo an endoscopic mucosal resection (EMR) of previously diagnosed lesions underwent NIR-FME imaging (Figure 1A). Five patients received an intravenous bolus of bevacizumab-800CW, two days prior to the procedure (4.5 mg, bolus injection). In addition, nine patients received topical administration of the tracer, by spraying bevacizumab-800CW on the luminal surface (100 µg/ml per cm BE) immediately prior to wide-field NIR-FME. Table 1 summarizes study subjects and lesion characteristics.
Detection of Esophageal Neoplasia by Wide-field Fluorescence Molecular Endoscopy

**Approach 1 - Systemic tracer administration**
- Systemic tracer administration
- + Endoscopic mucosal resection
- 2 days

**Approach 2 - Topical tracer application**
- Topical tracer spraying
- Incubation time
- 5 min.

**Tracer:** bevacizumab-800CW
- Systemic: 4.5 mg (bolus injection) | n = 5
- Topical: 100 μg/ml per cm BE | n = 9

**Fluorescence intensity**
- Low
- High

**Figure 1. Real-time VEGFA targeted NIR-FME.** (A) Schematic overview and timeline of the two NIR-FME approaches. (B) Representative images of the systemic tracer based, and (C) the topical tracer based wide-field NIR-FME results. (D) Additionally identified fluorescent areas, not visible during HD/NBI inspection. All fluorescence signals presented are uncorrected; overlay images display the high intensities only. NIR-FME, near-infrared fluorescence molecular endoscopy; HD, high-definition; EAC, esophageal adenocarcinoma; LGD, low-grade dysplasia.
All endoscopic procedures were performed by BE expert gastroenterologists. HD-WL inspection and narrow-band imaging (NBI) of the complete BE segment was first performed to confirm the presence of previously diagnosed dysplastic and neoplastic areas scheduled for EMR treatment. Subsequently, the entire BE segment was inspected with NIR-FME. At the end of endoscopic surveillance, all suspicious lesions were resected under endoscopic guidance. Control biopsies were obtained 8-12 weeks post-EMR during follow-up endoscopy.

**NIR-FME following systemic tracer administration**

HD-WL endoscopy identified a single EAC lesion in each patient from the group of five patients that received systemic administration of the fluorescence tracer. Four of these EAC lesions were also identified by wide-field NIR-FME inspection (Figure 1B). The fifth lesion had its origin in the gastric cardia and was only visible in endoscopic U-turn (Suppl. Figure 1A). Due to the bending restrictions of the current fiber bundle employed, this lesion was not accessible for the custom-built NIR-FME endoscope employed in the study. Nevertheless, this fifth lesion demonstrated high fluorescent tracer uptake and VEGFA expression levels as identified by ex vivo analyses of the EMR specimen (Suppl. Figure 1B-C). The five lesions were protruding (n = 1), elevated (n = 2) or flat lesions (n = 2). The average in vivo target (tumor)-to-background ratio (TBR) obtained by systemic tracer administration was 2.75 ± 0.29 (Suppl. Figure 2A).

**NIR-FME following topical tracer administration**

For the group receiving topical tracer administration, eight out of nine patients had previously identified focal lesions using HD-WL endoscopy. Three patients had two aberrant lesions. For the ninth patient, no focal lesion could be visualized during HD-WL and NBI inspection; an adenocarcinoma was previously identified based on sampled random biopsies only.

Topical tracer based NIR-FME visualized all twelve dysplastic and neoplastic lesions (Figure 1C), including the suspicious area that was only identified by random biopsies. This latter fluorescence-positive lesion, which was missed by HD-WL and NBI inspection, was shown to contain adenocarcinoma on the histopathological examination of the EMR specimen (pT1m2). The fluorescent signals outlining the twelve focal lesions could be visualized within seconds following the topical application of the tracer (Figure 2).

Moreover, topical tracer based NIR-FME identified four additional fluorescence-positive lesions not identified by HD-WL and NBI inspection. Histopathological examination of biopsies taken from these positive lesions confirmed the presence of three dysplastic areas and one area that contained atypical crypts, diagnosed as indefinite for dysplasia (Figure 1D). The topical administration led to a slightly higher in vivo TBR of 4.30+/-.0.41, compared to the systemic administration (Suppl. Figure 2B).
Adverse events

We observed adverse events (AE) in 4/14 patients, though all known complications of the EMR procedure: bleeding (n = 2), nausea/headache (n = 1) and fever (n = 1). The two bleeding complications developed within 24 hours after the procedure, originating from the site of the EMR (systemic group: arterial bleed, topical group: venous bleed). Both bleeds were successfully treated endoscopically. The nausea, headache and fever arose the day after the procedure (both in topical group) and resolved spontaneously within a few hours.

These events are most likely not tracer-related since only 1-1.5% of the therapeutic bevacizumab dose was used (4.5 mg vs. 5 mg/kg). The Data Safety Monitoring Board (DSMB) and ethical committee judged that the tracer was safe for both topical and systemic use. Since bleeding complications are commonly reported following EMR procedures,\textsuperscript{22-26} especially when patients are on anticoagulant therapy, they concluded that the two bleeding events that occurred during our study were most likely not related to the tracer or to the NIR-FME technique.
Histopathology
The 21 lesions that were resected (or biopsied) from our study subjects (i.e. 5 lesions from the group receiving systemic tracer administration, 11 lesions identified both by HD-WL and NIR-FME in the group receiving topical tracer administration and the 5 additional dysplastic lesions identified only by NIR-FME) were submitted for routine histopathology and IHC analysis. All 21 resected lesions were confirmed dysplastic or neoplastic (Table 1). Moreover, all 21 lesions were found to overexpress VEGFA (Figure 3A). All control biopsies, obtained 8-12 weeks post-EMR, revealed no new dysplastic areas. This finding preliminary suggests an absence of false negatives in the FME surveillance performed in the patient sample.

These findings are in line with an anti-VEGFA immunohistochemistry (IHC) validation assay, which was performed on additional EMR specimens and esophageal biopsies (n = 85; see supplemental methods). These IHC results demonstrated that all dysplastic and neoplastic tissues expressed VEGFA. ~82% of the lesions examined demonstrated high staining intensity and ~12% of the lesions showed intermediate staining intensity (Suppl. Figure 2). In contrast, the benign esophageal specimens showed low to no staining.

Ex vivo tissue analyses
Ex vivo assessment of the specimen obtained from the group receiving systemic tracer administration showed significant differences in mean fluorescence intensity (MFI) between the aberrant areas (EAC/HGD) and the benign mucosal lining, resulting in a favorable ex vivo TBR of 16.7 (Figure 3B, P value < 0.001). Furthermore, ex vivo NIR microscopy of the formalin-fixed and paraffin embedded (FFPE) slices confirmed the presence of NIR fluorescence in EAC areas (Figure 3C), tumor stromal tissue and surrounding tumor blood vessels (Figure 3D-E).

Likewise, macroscopic ex vivo imaging of the formalin-fixed specimens that received topical tracer administration exhibited NIR fluorescence at dysplastic and EAC sites (Suppl. Figure 3), though typically at lower intensity compared to the samples from the group receiving systemic administration due to agent washout during the fixation process. Ex vivo fluorescence imaging was performed using an epi-illumination wide-field fluorescence imaging system (LI-COR Biotechnology, Lincoln, NE, USA; see methods for details)
### Table 1. Patient characteristics

<table>
<thead>
<tr>
<th>Participants, no. (%)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>12 (80%)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>2 (20%)</td>
<td></td>
</tr>
<tr>
<td>IV tracer administration</td>
<td>5 (36%)</td>
<td></td>
</tr>
<tr>
<td>Topical tracer application</td>
<td>9 (64%)</td>
<td></td>
</tr>
</tbody>
</table>

| Age, mean (range), years | 65.07 (48-77) |  |
|--------------------------|-----------------|  |

| Body mass index (BMI), mean ± SD | 27.67 ± 5.52 |  |
|-------------------------------|--------------|  |

<table>
<thead>
<tr>
<th>BE segment mean (range), cm, Prague criteria</th>
<th>3.64 (0 – 10)</th>
<th>5.57 (1 – 13)</th>
</tr>
</thead>
</table>

| Focal lesion description no. (%); Paris classification | 17* |  |
|--------------------------------------------------------|-----|  |
| Protruding lesion (Is) | 5 (29%) |  |
| Elevated lesion (Iia) | 3 (18%) |  |
| Flat lesion (Ilb) | 8 (47%) |  |
| Unidentifiable focal lesion during HD/NBI | 1 (6%) |  |

**Aberrant tissue identified with NIR-FME**

| Fluorescent focal lesions, no. (%) | 16/17 (94%) ** |  |
|------------------------------------|----------------|  |
| IV tracer administration | 4/5 (80%) ** |  |
| Topical tracer application | 12/12 (100%) |  |

| Histological grade, no. (%) | 17* |  |
|----------------------------|-----|  |
| Focal lesions: |  |
| EAC | 15 (88%) |  |
| HGD | 1 (6%) |  |
| LGD | 1 (6%) |  |

<table>
<thead>
<tr>
<th>Additional fluorescent areas, no. (%)</th>
<th>5</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>IV tracer administration</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Topical tracer application</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Histological grade, no. (%)</th>
<th>5</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Additional lesions:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EAC</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>HGD</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>LGD</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Indefinite for dysplasia</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

**NOTE.** *In three out of 14 patients two separate focal lesions were present, resulting in 17 focal lesions in total. **One lesion was not identifiable during IV NIR-FME: gastric cardia lesion, only visible in U-turn, which is not feasible with NIR-FMEE system. Abbreviations: NIR-FME, near-infrared Fluorescence Molecular Endoscopy; HD, high-definition; NBI, narrow-band imaging; EAC, esophageal adenocarcinoma; HGD, high-grade dysplasia; LGD, low-grade dysplasia; IV, intravenous.
Figure 3. *Ex vivo* VEGFA and fluorescent signal analyses of study subjects. (A) Boxplot presenting IHC results (H-score: median, 10-90 percentile); a significant difference in VEGFA staining intensities is observed between the dysplastic and benign tissue sites. **P < 0.0001. (B) Tukey boxplot presenting the MFIs observed (mean value in bold). *P < 0.05. (C) Fluorescence scan and 3D surface-plot demonstrating that the tracer is located in the EAC area, and (D) fluorescence microscopy of this same area showing NIR fluorescence within the stromal tissue, between the tumor ducts. (E) Fluorescence scan demonstrating that the tracer is located in tumor blood vessels and tumor ducts. IHC, immunohistochemistry; EAC, esophageal adenocarcinoma; HGD, high-grade dysplasia; IM, intestinal metaplasia; MFI, mean fluorescence intensities; NIR, near-infrared.
DISCUSSION

We hypothesized that fluorescence endoscopy using near-infrared agents can serve as a real-time red-flag technique for dysplasia and neoplasia identification in Barrett esophagus (BE). We examined whether a large-antibody based fluorescent tracer targeted against VEGFA could be employed for NIR-FME of early cancerous and dysplastic lesions of the esophagus in BE patients. To corroborate the findings, we further compared systemic vs. topical administration using the same collection system and agent. NIR-FME results demonstrate previously undisclosed detection of BE neoplasia and dysplasia in real-time, i.e. during the endoscopic procedure. We show that although HD-WL and NBI detected 16 lesions, fluorescence molecular imaging could identify 20 lesions, all confirmed histologically as dysplastic or malignant. To the best of our knowledge this is the first demonstration of the addition of FME potential to surpass the performance of white-light imaging and therefore could improve future BE surveillance strategies.

To achieve detection of systemic agent administration under microdosing conditions, we employed highly sensitive endoscopic imaging using a technology that was previously developed for ultra-sensitive detection in breast cancer surgery. We demonstrated that detection of aberrant lesions in the near-infrared spectrum is possible through a miniaturized scope operating in a mother-daughter configuration around a commercially available HD-WL endoscope.

Regarding the preference for a topical or systemic tracer administration, we found that both approaches—with either a topical or systemic tracer—are feasible and can be used to identify early aberrant esophageal lesions in real-time. Based on our data, although derived from a small number of procedures, topical tracer administration would be preferred for diagnostic purposes since it does not impact the standard workflow of the gastroenterologist. Moreover, it showed higher in vivo TBR signals over systemic administration. This can be explained by the fact that topical administration delivers higher amounts of tracer to the lesion, over the volumetric distribution of systemically administered agents. Moreover, two-dimensional fluorescence imaging is surface-weighted, i.e. detects superficial signals with high sensitivity; therefore it is ideally suited for surface-based agent delivery.

On the other hand, systemic agent administration may prove necessary in alternative NIR-FME applications. For example the evaluation of endoscopic treatment accuracy requires inspection for aberrant cells infiltrating deeper in sub-surface tissues that are not accessible by topical administration of tracers (Suppl. Figure 3). In addition, systemic administration would be of interest to gain insight into drug distribution of targeted agents for esophageal cancer treatment.
Ex vivo fluorescence-signal analyses and VEGFA IHC confirmed elevated fluorescence congruent with high intracellular VEGFA stainings, which was in line with our validation of VEGFA expression in Barrett samples (Suppl. Figure 4). This finding is consistent with previous preclinical and clinical observations linking bevacizumab and labeled-bevacizumab uptake to areas of elevated VEGFA. Moreover, multiple mouse studies using competition experiments based on scrambled bevacizumab and VEGF ELISA analyses demonstrated highly selective VEGFA-driven uptake of labeled bevacizumab in tumor models. After intracellular VEGFA production, the protein is excreted in the extracellular matrix and part of its large isoforms will bind with high affinity to heparin sulphate proteoglycans (HSPGs). It has been observed that Bevacizumab or labeled bevacizumab, when bound to the large VEGFA isoforms attached to the cell surface, is trapped in the extracellular matrix as described before in the context of $^{89}$Zr-bevacizumab.

In recent years, the quest for early esophageal lesion identification, combined with the known limitations of the current commercially available endoscopic methods, has led to a continuous search for new and better imaging techniques such as molecular imaging. To date, three studies—one ex vivo study and two clinical trials—have been reported on FME in EAC; though, none of these studies showed real-time wide-field imaging results. Compared to previous studies, the NIR-FME technique offers four major advantages. First, the system design enabled concurrent use of high-definition white-light endoscopy and ultra-sensitive functional fluorescence detection of lesions, allowing detection of micro-dosing amounts of administered agent. Second, despite the promise of confocal laser endomicroscopy (CLE), BE surveillance requires a wide-field inspection technique. NIR-FME offers a ‘red-flag’ surveillance method over standard morphology information obtained by white light imaging, that can be further exploited to guide biopsy, CLE and potentially treatment. Third, NIR-FME allowed easy real-time identification of BE lesions, a feature never achieved before for FME. Finally, we showed for the first time that NIR-FME following topical administration of the tracer could identify ~33% more lesions, missed by HD-WL and NBI. A key advance that allowed these advantageous characteristics relates to the switch from visible wavelengths to the NIR. Compared to previous studies using fluorophores emitting in the visible, NIR tracers enable parallel operation with white light imaging and lead to more sensitive fluorescence detection. Moreover, NIR wavelengths penetrate deeper into the target tissue and are less subjected to interference by hemoglobin absorption, autofluorescence signals and tissue scattering. Future software improvements could even automatically alert the endoscopist and thereby may reduce the human factor.

In conclusion, this proof-of-principle study demonstrates that concurrent use of VEGFA-guided NIR-FME and HD endoscopy, following topical or systemic tracer administration,
can be used to detect dysplastic and early EAC lesions in BE patients. Our in vivo NIR-FME results show that even flat and difficult to distinguish lesions were identifiable with NIR-FME. Moreover, the topical tracer approach was able to improve early lesion detection with ~33% compared to WL endoscopy. In addition, our NIR-FME approach can be used in standard clinical practice since it adds real-time molecular guidance, and thus functional information, without interfering with the regular HD morphology information of white-light endoscopy. Although this report illustrates the potential of endoscopic wide-field NIR-FME as an aid to improve esophageal lesion detection, further research that involves a larger study population is required to verify the additive role of NIR-FME in BE surveillance and treatment strategies.

METHODS

Near-infrared Fluorescence Molecular Endoscope

A NIR-FME system was custom-built around an existing CE-marked clinical grade HD endoscope. A Micrendo® fiber-bundle containing 30,000 coherently arranged individual fibers (Scholly Fiberoptic GmbH, Denzlingen, Germany) was connected to a clinical prototype camera system (SurgVision BV, Heerenveen, The Netherlands), as shown in the schematic diagram of the setup (Figure 4). The fiber-bundle (daughter scope) has a field-of-view of 85° and its physical dimensions allow it to be inserted through the accessory channel of routine gastrointestinal video endoscopes (mother scopes). This mother-daughter approach minimizes impact on the current workflow, as it can be inserted through a conventional HD white-light endoscope. The NIR-FME system is equipped with a LED light source for white-light illumination and detection through a short pass filter (< 750 nm; E700SP-2P, Chroma, Bellows Falls, VT, USA) and a laser (750 nm, BWF1, B&WTEK, Newark, DE, USA) for fluorescence excitation. The connection of the miniature fiber-bundle to the camera system is made feasible via a mechanical and focusing adapter (Scholly Fiberoptic GmbH, Denzlingen, Germany), while a multi-branched fiber-optic bundle (SEDI-ATI Fibres Optiques, Courcouronnes, France) provides simultaneous white-light illumination and fluorescence excitation coupling. To assure fluorescence detection within the 800 nm range, the NIR camera system is further equipped with a band pass filter (819 ± 44, Semrock inc, Rochester, NY, USA). The system is specifically developed for highly sensitive imaging, allowing video-rate detection of fluorochromes administered in microdosing amounts.
Figure 4. Schematic overview of the NIR-FME system. This figure illustrates attachment of the fiber-bundle to an external ultra sensitive NIR fluorescence camera and insertion through the working channel of a clinical HD video-endoscope. EAC, esophageal adenocarcinoma; NIR, near-infrared; FME, fluorescence molecular endoscopy; HDE, high-definition endoscope; WL, white-light; BPF, band pass filter (819 nm, ±44 nm); SPF, short pass filter (<750 nm).

Near-infrared (NIR) fluorescent tracer: bevacizumab-800CW

NIR-FME was based on the previously reported anti-VEGF targeted near-infrared (NIR) fluorescent tracer consisting of NIR fluorophore IRDye 800CW (IRDye 800CW NHS ester; LI-COR Biosciences, Lincoln, NE, USA) conjugated to the monoclonal antibody bevacizumab (Avastin, Roche, Hertfordshire, United Kingdom). The agent has been already employed in preclinical and clinical studies. Clinical grade bevacizumab-800CW was produced at the GMP facility of the University Medical Center Groningen (UMCG, Groningen, the Netherlands). In this procedure, the IRDye 800CW was bound to lysine amino acids of the bevacizumab protein backbone in a phosphate-buffered saline solution (pH 8.5); a dye-to-protein molar ratio of 4:1 was used for the intravenous group and a 2:1 ratio for the topical administration group. After conjugation of the dye molecules to the antibody, the tracer product was purified by buffer exchange, formulated and filled into injection vials [1mg/ml]. During and after production, quality control was performed to assess purity, quality, sterility and biological activity of the tracer. Labeling efficiency, quality and purity were determined by...
Detection of Esophageal Neoplasia by Wide-field Fluorescence Molecular Endoscopy

Endoscopic study in BE patients

The study was run as a proof-of-principle clinical trial. Eligibility for inclusion implied the presence of histologically confirmed HGD or superficial EAC (TNM classification: ≤ cT1). In addition, only patients undergoing EMR therapy were included. Exclusion criteria excluded all patients with medical or psychiatric conditions that would compromise their ability to give informed consent, concurrent medical conditions that would disqualify the patient for undergoing the endoscopic mucosal resection (EMR) procedure and the presence of pregnancy or breast-feeding at the time of participation.

The study was carried out as a single center, open-label, non-randomized, investigator-initiated clinical trial. The Medical Ethics Committee of the University Medical Center Groningen (UMCG, Groningen, the Netherlands) approved the trial, which was registered at clinicaltrials.gov (NCT02129933) prior to initiation. All included patients (n = 14) provided written informed consent prior to start of the trial.

NIR-FME was performed by experienced gastroenterologists (F.T.M.P; W.B.N) concurrently with HD-WL endoscopy and within the same endoscopic session as the EMR procedure. Figure 1A illustrates the NIR-FME study design: nine study subjects received the tracer topically (approximately 1 ml per cm BE segment) and five study subjects received the tracer via an intravenous bolus injection two days prior to the endoscopic procedure. For the topical group, NIR-FME was performed at several time-points: 1) at baseline (after cleaning with acetyl cysteine 0.1%), 2) immediately after application of the tracer (images presented in Figure 1C), and 3) following incubation (5 minutes) and rinsing off the excess tracer with water. In addition to the specimen obtained by EMR, biopsies were taken from non-dysplastic Barrett and normal squamous mucosa and from areas exhibiting increased fluorescence on the NIR-FME recordings.
**Ex vivo analyses of the fluorescence signals**

To confirm the in vivo fluorescent signals, we performed NIR fluorescence macroscopic and microscopic imaging of all ex vivo samples obtained in the study.

Macroscopic fluorescence imaging was performed with the Odyssey® CLx infrared flatbed scanner (LI-COR Biotechnology, Lincoln, NE, USA) using 785 nm excitation on 1-2 mm thick formalin-fixed EMR slices. The slices were scanned at a fixed intensity (intensity 6) and image resolution of 21 µm. Subsequently the formalin-fixed slices were embedded in paraffin for routine histopathology.

For achieving more detailed (higher-resolution) specimen interrogation we cut 10 µm thick sections from the 1-2mm slices. The 10 µm sections were deparaffinized in xylene for 10 minutes and imaged with the fluorescence flatbed scanner, now at intensity 9 and image resolution of 21 µm. Then, they were stained with hematoxylin and eosin (HE) for corresponding morphologic evaluation of the analyzed tissue. Mean fluorescence intensities (MFIs) per region of interest (ROI) were calculated in ImageJ (Vers. 1.48, U. S. National Institutes of Health, Bethesda, MD, USA). Tumor-to-Background ratios (TBRs) were estimated as the quotient of the MFI in the aberrant tissue over adjacent or biopsied benign tissue (squamous and IM).

Additionally, 4 µm sections were cut from the 1-2mm thick slices for microscopic NIR fluorescence analysis. The slices were counter-stained with Hoechst staining, (33258; Invitrogen, Carlsbad, CA, USA) and cover slips were mounted with Kaiser’s glycerine (modified). Microscopy was performed using an inverted laser-scanning microscope (DMI6000B, Leica Biosystems GmbH, Nussloch, Germany) equipped with a highly sensitive monochrome DFC365 FX fluorescence camera (1.4M Pixel CCD, Leica Biosystems GmbH, Nussloch, Germany), an optimal NIR filter set (two band-pass filters 850-90m-2p and a long-pass emission filter HQ800795LP; Chroma Technology corp®, Bellows Falls, VT, USA), a highly sensitive NIR LED light source ranging up to 900 nm (X-Cite® 200DC, Excelitas™ technologies, Waltham, MA, USA) and LAS-X software (Leica Biosystems GmbH, Nussloch, Germany).

**Confirmatory immunohistochemistry**

VEGFA IHC stainings were performed as described previously. Although we only had access to an IHC antibody that stained the intracellular isoform of VEGFA, this antibody can be used to estimate its extracellular presence, since most likely an equilibrium is present between the intracellular and extracellular isoforms. In brief, 4 µm tissue sections were cut, mounted on slides, deparaffinised in xylene and rehydrated in a series of ethanol dilutions. Secondly, heat-induced antigen retrieval was conducted using a 100mM Tris-HCL buffer (pH 9) followed by blocking of the endogenous peroxidase activity by incubation with 30%
hydro-peroxide diluted in phosphate buffered saline (PBS). Tissue sections were incubated at room temperature for 15 minutes with an Avidin/Biotin blocking kit (SP-2001, Vector Laboratories, Burlingame, CA, USA). Subsequently, the primary antibody against VEGFA (A-20; clone sc-152, rabbit polyclonal IgG, Santa Cruz Biotechnology, Dallas, TX, USA) was added at room temperature, for 1 hour at a 1:50 dilution in PBS. Afterwards, the second and third antibody, respectively biotinylated swine anti-rabbit and Streptavidin/HRP (Dako, Glostrup, Denmark), were incubated for 30 minutes at a dilution of 1:300 in PBS, including 1% bovine serum albumin (BSA) and 1% human AB-serum. Finally, specific staining was visualized using 3,3’ dianinobenzidine (DAB) substrate chromogen solution and counterstaining of the nuclei by use of hematoxylin.

IHC staining protocols were optimized for their applicability in esophageal tissue. To ascertain specific binding of the antibody, positive and negative controls were included in OACH stained sample batch. EAC, dysplasia, intestinal metaplasia and adjacent normal tissue were scored for staining intensity (0-3 scale) and the percentage of cells stained. This visual scoring was performed separately by two observers (E.H., W.B.v.E.). Subsequently, H-scores were generated (continuous scale: 0-300) by combining the evaluated intensity and the corresponding percentage of cells stained (formula used: $1 \times \text{percentage of cells weakly stained \[1^+\]} + 2 \times \text{percentage of cells moderately scored \[2^+\]} + 3 \times \text{percentage of cells strongly stained \[3^+\]}$), creating expression categories representative for overall protein expression per tissue type (0-100 = negative/low; 101-200 = intermediate; 201-300 = high).

**Statistics**

For statistical analysis, IBM SPSS 22.0 was used (IBM Corporation, Armonk, NY, USA). Our ex vivo data was analyzed according to distribution and sample-size, using a non-parametric Kruskal-Wallis test, corrected for multiple testing. $P$ values < 0.05 were considered statistically significant.

**ACKNOWLEDGEMENTS**

We would like to thank research analyst W. Boersma-van Ek for her assistance in the laboratory, especially for her contribution in the IHC staining and scoring process. Furthermore, we would like to thank Prof. J.J.G.H.M. Bergman for carefully reading the manuscript.
COMPEETING INTERESTS

W.B.N. and G.M.v.D. received an unrestricted research grant for molecular imaging development from SurgVision BV (the Netherlands). G.M.v.D. and V.N. are in the scientific advisory board of Surgvision BV The other authors have no conflicts to declare.

FUNDING

This work was financially supported by the Dutch Cancer Society (RUG 2012-5416) and via an unrestricted research grant for molecular imaging from SurgVision BV (The Netherlands).
REFERENCES


**Supplementary Materials**

**Suppl. Figure 1. Gastric cardia lesion.** (A) This lesion had its origin in the cardia of the stomach and was only visible in endoscopic U-turn. Endoscopic U-turn cannot be accomplished with NIR-FME due to bending restrictions of the fiber-based system. Therefore, the fluorescence signal of this lesion could not be assessed with NIR-FME. (B) These images shows that the cardia lesion contained EAC, which strongly expressed VEGFA. (C) During ex vivo signal analyses, the formalin-fixed section that contained EAC expressed high fluorescence; normal mucosal lining showed a negligible fluorescence intensity (black dotted line). EAC, esophageal adenocarcinoma; HD, high-definition; EMR, endoscopic mucosal resection; NIR-FME, Near-infrared Fluorescence Molecular endoscopy; HE, hematoxylin and eosin; VEGFA, vascular endothelial growth factor A.

**Suppl. Figure 2. Imaging statistics of topical vs. systemic FME signal.** This figure illustrates the in vivo TBR for the different labeling approaches. (A) The systemic injection of bevacizumab-800CW lead to a TBR of 2.75 ± 0.29. (B) The topical application resulted in a TBR of 4.30 ± 0.41 (mean ± std) prior to rinsing. The images are normalized to match a mean of 1 in the surrounding tissue region of interest. The scattered plot shows the statistics for the surrounding tissues (bottom cluster) and tumor region (upper cluster). The black dots represent single intensity values. The red lines show the mean for each group. The one standard deviation area is colored in blue. The data is jittered and subsampled for visualization. TBR, tumor-to-background ratio.
Part 1  Chapter 4

Supplemental Target Validation

Immunohistochemistry (IHC) was performed to examine the expression of vascular endothelial growth factor A (VEGFA) in dysplastic and neoplastic oesophageal BE derived lesions. Tissue samples of 85 different individuals with BE, including endoscopic mucosal resection (EMR) derived specimens and biopsies, were selected from an existing BE database (University Medical Center Groningen, collected between 1995-2012) and immunohisto-stained for the presence of VEGFA (OAC n = 21; high grade dysplasia HGD n = 26; intestinal metaplasia n = 41; squamous epithelium n = 70). Similarly, EMR and biopsy material derived from subjects undergoing the NIR-FME procedure were collected and immunohisto-stained for the presence of VEGFA (OAC n = 21; high grade dysplasia HGD n = 26; intestinal metaplasia n = 41; squamous epithelium n = 70). What can be appreciated from this figure is that, despite only a limited amount of fluorescent tracer remained in the topical tracer based specimens after formalin fixation (A: only superficial spots), compared to the systemic tracer based specimens can be observed (B: fluorescence within the deeper tissue layers). Since the amount of tracer present within the systemic specimen is much higher, enough signal is left to accomplish ex vivo analyses.

Suppl. Figure 3. Fluorescence in topical tracer specimen. A clear difference in the amount of tracer left in the topical tracer based specimens after formalin fixation (A: only superficial spots), compared to the systemic tracer based specimens can be observed (B: fluorescence within the deeper tissue layers). Since the amount of tracer present within the systemic specimen is much higher, enough signal is left to accomplish ex vivo analyses. What can be appreciated from this figure is that, despite only a limited amount of fluorescent tracer remained in the topical tracer based specimens following fixation, the fluorescent spots that are still present are within the sections that contained aberrant tissue, located at sites of dysplastic and neoplastic cells, therefore signifying the specificity of the tracer (indicated with rectangles; confirmed by HE). The benign sections (squamous, IM) did not contain these high fluorescent spots. EAC, oesophageal adenocarcinoma; HGD, high-grade dysplasia; LGD, low-grade dysplasia; IM, intestinal metaplasia; HE, haematoxylin and eosin.

Suppl. Figure 4. Ex vivo target validation: anti-VEGFA IHC.

Box plot (median, 10-90 percentile) and bar graph, both presenting VEGFA IHC results (H-score) in esophageal samples, including esophageal adenocarcinoma, dysplasia, intestinal metaplasia and normal squamous epithelium. The representative images illustrate the significant difference in VEGFA-staining intensities (brown); the high staining intensity observed in the aberrant lesions (EAC and dysplasia) is in clear contrast with the low to negative stained benign tissue (incl. IM and squamous epithelium). IHC, immunohistochemistry; EAC, esophageal adenocarcinoma; IM, intestinal metaplasia. ***P < 0.0001 (Kruskal-Wallis test).
SEARCH FOR NOVEL MOLECULAR TARGETS