

Research Paper

# Potential Red-Flag Identification of Colorectal Adenomas with Wide-Field Fluorescence Molecular Endoscopy

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## Abstract

Adenoma miss rates in colonoscopy are unacceptably high, especially for sessile serrated adenomas / polyps (SSA/Ps) and in high-risk populations, such as patients with Lynch syndrome. Detection rates may be improved by fluorescence molecular endoscopy (FME), which allows morphological visualization of lesions with high-definition white-light imaging as well as fluorescence-guided identification of lesions with a specific molecular marker. In a clinical proof-of-principal study, we investigated FME for colorectal adenoma detection, using a fluorescently labelled antibody (bevacizumab-800CW) against vascular endothelial growth factor A (VEGFA), which is highly upregulated in colorectal adenomas.

**Methods:** Patients with familial adenomatous polyposis (n = 17), received an intravenous injection with 4.5, 10 or 25 mg of bevacizumab-800CW. Three days later, they received NIR-FME.

**Results:** VEGFA-targeted NIR-FME detected colorectal adenomas at all doses. Best results were achieved in the highest (25 mg) cohort, which even detected small adenomas (<3 mm). Spectroscopy analyses of freshly excised specimen demonstrated the highest adenoma-to-normal ratio of 1.84 for the 25 mg cohort, with a calculated median tracer concentration in adenomas of 6.43 nmol/mL. *Ex vivo* signal analyses demonstrated NIR fluorescence within the dysplastic areas of the adenomas.

**Conclusion:** These results suggest that NIR-FME is clinically feasible as a real-time, red-flag technique for detection of colorectal adenomas.

Key words: optical molecular imaging, spectroscopy, vascular endothelial growth factor a, near-infrared fluorescence.

## Introduction

Colorectal cancer (CRC) is the second most common cancer worldwide, accounting for 8% of all cancer related deaths [1]. Of all CRCs, approximately 60% develops via the well-known adenoma-carcinoma sequence, one-third via the alternative serrated pathway and a small portion from Lynch syndrome (LS) [2]. White-light endoscopy is considered the gold standard for detection and removal of colorectal lesions, to prevent CRC development. However, detection of small adenomas (<5 mm) and sessile serrated adenomas / polyps (SSA/P) is difficult since conventional endoscopy relies upon aspecific morphological tissue signatures and thus on the experience of the endoscopist. As a result, the reported adenoma detection miss rate for the general population is relatively high (27%) [3]. For patients with LS, adenoma miss rates are even up to 55%. In this high-risk population small adenomas are more common, and these often already contain high-grade dysplasia (HGD) since the adenoma-carcinoma sequence is known to be accelerated [4,5]. Therefore, missed lesions can rapidly progress to cancer which results in an unacceptable cumulative cancer risk of up to 35% at the age of 60, despite intensive screening programs. This underscores the necessity of improving endoscopic detection strategies [6].

One way to improve endoscopic lesion identification is the incorporation of wide-field fluorescence molecular endoscopy (FME). FME visualizes lesions based on their biological properties rather than their morphology; it uses exogenous fluorescent tracers that bind to specific proteins, thereby fluorescently 'highlighting' the tissue of interest as a 'red-flag' for the endoscopist. A recently published study showed a higher adenoma detection rate with FME following an intravenous (IV) injected anti-cMET tracer using an old white-light fiber endoscope for both fluorescence and white-light imaging, which hampers clinical translation [7]. Moreover, FME 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 also reduces significantly the transmission of fluorescence signals. Moreover, the visible light exhibits strong autofluorescence, reducing contrast.

Therefore, we labelled the monoclonal antibody bevacizumab with a near-infrared (NIR) fluorescent dye, IRDye 800CW, and used a NIR-FME platform that enables concurrent fluorescence and

high-definition (HD) white-light imaging. Bevacizumab-800CW binds vascular endothelial growth factor A (VEGFA), which is present in all stages of colorectal neoplasms, including low grade dysplastic (LGD) adenomas and in up to 90% of difficult to detect but clinically important sessile serrated adenomas/polyps (SSAPs) [8,9]. We evaluated VEGFA-targeted NIR-FME for adenoma detection in a dose-escalation study, performed in patients with familial adenomatous polyposis (FAP) who have a high probability of occurrence of colorectal adenomas. We choose FAP patients due to the abundance of colorectal adenomas in this condition, to enable adequate evaluation of the different dose steps. To validate our *in vivo* NIR-FME findings, we quantified the fluorescence of excised colorectal tissue by correcting for the influence of tissue optical properties using Multi-Diameter Single Fiber Reflectance and Single Fiber Fluorescence (MDSFR/SFF) spectroscopy (Figure 1).

## Materials and Methods

### Study population and design

Patients with FAP that were 18 years of age or older, and scheduled for surveillance endoscopy at the University Medical Center Groningen (UMCG), were invited to participate in the study. Trial enrolment required FAP to be genetically proven or clinically diagnosed by >100 colorectal adenomatous polyps at earlier endoscopy. Patients with a *MutY* human homolog gene (*MUTHY*) mutation or who had a proctocolectomy were excluded. The study protocol was approved by the Medical Ethics Committee of the UMCG. All patients gave their written informed consent for participation in the study before inclusion. The study was registered with ClinicalTrials.gov (NCT02113202).

This non-randomized, non-blinded, single center proof-of-principle study consisted of three tracer-dose cohorts: 4.5, 10 and 25 mg of bevacizumab-800CW (Figure 1A) [10]. These tracer dosages are low compared to the therapeutic dose of bevacizumab (5-10 mg/kg) [11]. Three days after IV tracer injection, patients underwent surveillance endoscopy with a HD endoscope followed by NIR-FME to visualize fluorescent signals. Afterwards, we validated the observed fluorescence by *ex vivo* signal quantification on fresh resected tissue with MDSFR/SFF spectroscopy. Additionally, to specify tracer distribution and enable correlation to histopathology, we performed NIR-fluorescence flatbed scanning, fluorescence microscopy and VEGFA immunohistochemistry (IHC) on formalin-fixed, paraffin-embedded (FFPE) tissue (Figure 1B).

In the 4.5 mg cohort, six FAP patients with adenomas were included. The interim analysis incorporated in our study protocol allowed for a tracer dose escalation if the 4.5 mg tracer dose appeared suboptimal. As a result, three patients were included in the subsequent dose cohorts (10 and 25 mg); the best-performing tracer-dose cohort (25 mg) was expanded to six FAP patients in total. All observed adverse events were noted until 72 h after intravenous tracer injection. This period was chosen as bevacizumab-800CW had shown a good safety profile in previous studies in patients with primary breast cancer and peritoneal carcinomatosis of colorectal origin [12].

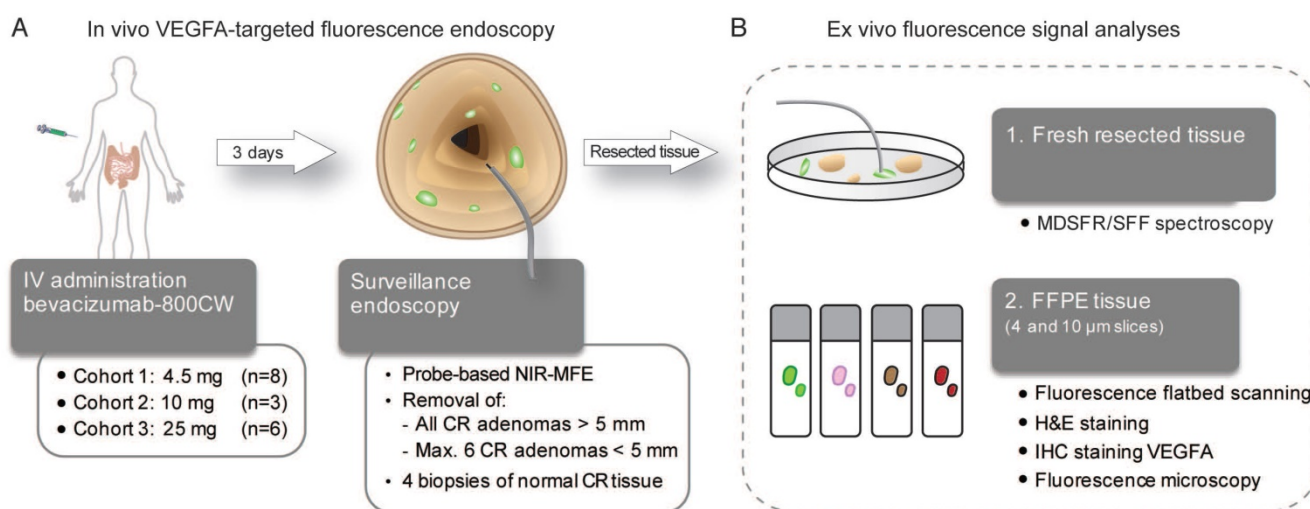
### NIR-FME procedure

Endoscopy was performed after standard bowel preparation, optionally under conscious sedation with midazolam and fentanyl. All procedures were performed with a routine clinical HD video endoscope, which is standard of care during surveillance endoscopies (CF-H180AL/I or GIF-H180J; EVIS EXERA II; Olympus Corporation, Tokyo, Japan). White light was provided by a standard xenon light source (CLV-180 Evis Exera II; Olympus Corporation), in which a short pass filter was installed (<750 nm, E700SP-2P; Chroma, Bellows Falls, VT, USA). When the caecum or ileorectal anastomosis was reached, the NIR-FME probe was introduced in the working channel of the video endoscope (online Figure S1A) [13]. During withdrawal, adenomas were concurrently visualized with both the video endoscope and the NIR-FME probe. The NIR-FME images (color, fluorescence and overlay) were

displayed on a separate monitor (Figure 2). Subsequently, all large adenomas ( $\geq 5$  mm; standard clinical care) and a maximum of six small adenomas (<3 mm) were excised. Additionally, four biopsies of normal appearing colorectal mucosa were taken for research purposes only. See supplementary materials for detailed information on tracer production and the technical background of the NIR-FME system.

### MDSFR/SFF spectroscopy

The freshly resected adenomas and normal mucosa biopsies were placed on ice with the mucosal side upwards. Directly after the endoscopy the MDSFR/SFF spectroscopy probe was placed on top of the fresh tissue for quantitative measurements of NIR fluorescence. This device gains two reflectance spectra via two different optical fibers and subsequently one raw fluorescence spectrum (Figure S1B and S2). From the reflectance spectra, the scattering and absorption coefficients were determined, which were used to determine the intrinsic fluorescence. The intrinsic fluorescence was afterwards used to calculate the actual bevacizumab-800CW concentration present in the fresh resected tissue. Subsequently, the majority of resected adenomas and normal tissue biopsies were formalin-fixed and paraffin-embedded (FFPE), while some were snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . In one patient (25 mg cohort) no quantitative measurements could be collected due to a technical malfunction of the MDSFR/SFF spectroscopy device. See supplementary materials for more detailed information on the MDSFR/SFF spectroscopy device, spectral fitting and determination of the local bevacizumab-800CW concentration.



**Figure 1. Study design.** (A) Intravenous administration of the fluorescent tracer bevacizumab-800CW, three days later followed by VEGFA-targeted fluorescence endoscopy. (B) Ex vivo fluorescent signal analyses: 1) quantification of the fluorescence signals with MDSFR/SFF spectroscopy, performed on fresh resected tissue, and 2) qualitative evaluation of tracer distribution performed on FFPE tissue. CR, colorectal; FFPE, formalin-fixed and paraffin-embedded; H&E, hematoxylin and eosin; IHC, immunohistochemistry; MDSFR/SFF, multi-diameter single fiber reflectance and single fiber fluorescence; NIR-FME, near-infrared fluorescence molecular endoscopy; VEGFA, vascular endothelial growth factor A.

## Histological fluorescence mapping

Per FFPE tissue block, one 10  $\mu\text{m}$  section and three 4  $\mu\text{m}$  sections were sliced, mounted on silane-coated slides and dried overnight at 37 °C. The 10  $\mu\text{m}$  FFPE tissue sections were deparaffinized (10 min xylene) and imaged with the NIR fluorescence flatbed. Afterwards, the 10  $\mu\text{m}$  tissue sections and the subsequent 4  $\mu\text{m}$  tissue sections were stained with hematoxylin and eosin (H&E). The H&E slides were digitalized by the Nanozoomer 2.0-HT slide scanner (Hamamatsu) and viewed with use of NanoZoomer Digital Pathology (NDP) viewer software (Hamamatsu). Blinded for the fluorescence signals and under supervision of an experienced gastrointestinal pathologist (A.K.), different tissue areas were selected: low-grade dysplasia (LGD) areas within the adenoma, normal adjacent tissue within the adenoma section and normal colon crypts within the normal tissue biopsies. Afterwards, these areas were superimposed on the NIR fluorescence Odyssey images. *Ex vivo* analyses of the snap frozen tissue was shown to be unreliable, as bevacizumab-800CW signals diminished during thawing of the samples.

## Fluorescence microscopy

For fluorescence microscopy, 4  $\mu\text{m}$  FFPE tissue sections were deparaffinized, rehydrated and stained with Hoechst to visualize nuclei (33258; Invitrogen, Thermo Fisher Scientific). Fluorescence microscopy was performed using an inverted wide-field microscope (63-100x magnification, immersion oil; DMI6000B, Leica Biosystems GmbH, Nussloch, Germany), with a LED light source that is able to excite up to 900 nm (X-Cite 200DC; Excelitas Technologies, Waltham, MA, USA), a monochrome camera also sensitive in the NIR range (1.4M Pixel CCD, DFC365FX; Leica Biosystems GmbH) and an adapted filter set (two band-pass filters 850-90m-2p and a long-pass emission filter HQ800795LP; Chroma Technology). All tissue slides were assessed using the same settings to enable visual comparison. Following acquisition, the images were processed with LAS-AF2 software (Leica Microsystems).

## Immunohistochemical analysis of VEGFA expression

We previously demonstrated the relevance of VEGFA as a target for colorectal neoplasia [8]. To determine if the prior VEGFA results hold true for our current patient population, we immunostained all FFPE colorectal tissue collected during this study (polyclonal rabbit anti-human VEGFA, RB9031 1:300; Thermo Fisher Scientific, Waltham, Ma, USA). To ascertain specific binding of the anti-VEGFA antibody, a positive tissue control and a negative IgG

control were included. Dysplastic crypts, normal crypts within the adenoma section and normal mucosa derived from the biopsies were scored separately for their staining intensity (0-3 scale) and the percentage of cells stained. This visual scoring was performed by two separate observers (E.H. and J.J.T.). Subsequently, H-scores were generated (continuous scale: 0-300) by combining the evaluated intensity and the corresponding percentage of cells stained [14]. See supplementary materials for detailed description of the IHC methods and H-score formula.

## Statistics

For statistical analysis of the MDSFR/SFF spectroscopy and flatbed scanning results IBM SPSS 22.0 (IBM Corporation, Armonk, NY, USA) and GraphPad Prism 5.0 (GraphPad Software Inc, La Jolla, CA, USA) were used. A two-tailed *t* test, Mann-Whitney U test or Kruskal-Wallis test was used, according to sample-size and distribution. *P* values <0.05 were considered statistically significant.

## Results

### Near-infrared fluorescence molecular endoscopy (NIR-FME)

In total, 17 patients participated in the study (Table 1). Eight patients received 4.5 mg, three patients 10 mg and another six patients 25 mg bevacizumab-800CW intravenously. No tracer-related adverse events were observed (0/17). Two patients in the 4.5 mg cohort did not have any lesions at endoscopy and were excluded from further analyses.

**Table 1.** Patient and adenoma characteristics

Number of patients, <i>n</i> (%)	17	
Complete colon <i>in situ</i>	5	(29.4%)
Ileorectal anastomosis	12	(70.6%)
Sex, <i>n</i> (%)		
Male	5	(29.4%)
Female	12	(70.6%)
Age, in years		
Median (range)	42	(20-65)
Total number of observed adenomas per patient, <i>n</i> (%)		
0	2 <sup>A</sup>	(11.8%)
1-5	4	(23.5%)
6-20	10	(58.8%)
>20	1	(5.9%)
Histology of resected lesions, <i>n</i> (%)	51	(100%)
LGD adenomatous polyp	50	(98%)
HGD adenomatous polyp	1	(2%)

<sup>A</sup>The two patients without any adenomas at endoscopy were left out of the *ex vivo* fluorescent signal analyses.

For all three tracer-dose cohorts, VEGFA-targeted NIR-FME fluorescently visualized all adenomas identified with white-light HD inspection concurrently (sensitivity 100%) (Figure 2). All larger adenomas ( $\geq 5$  mm) showed sufficient fluorescent



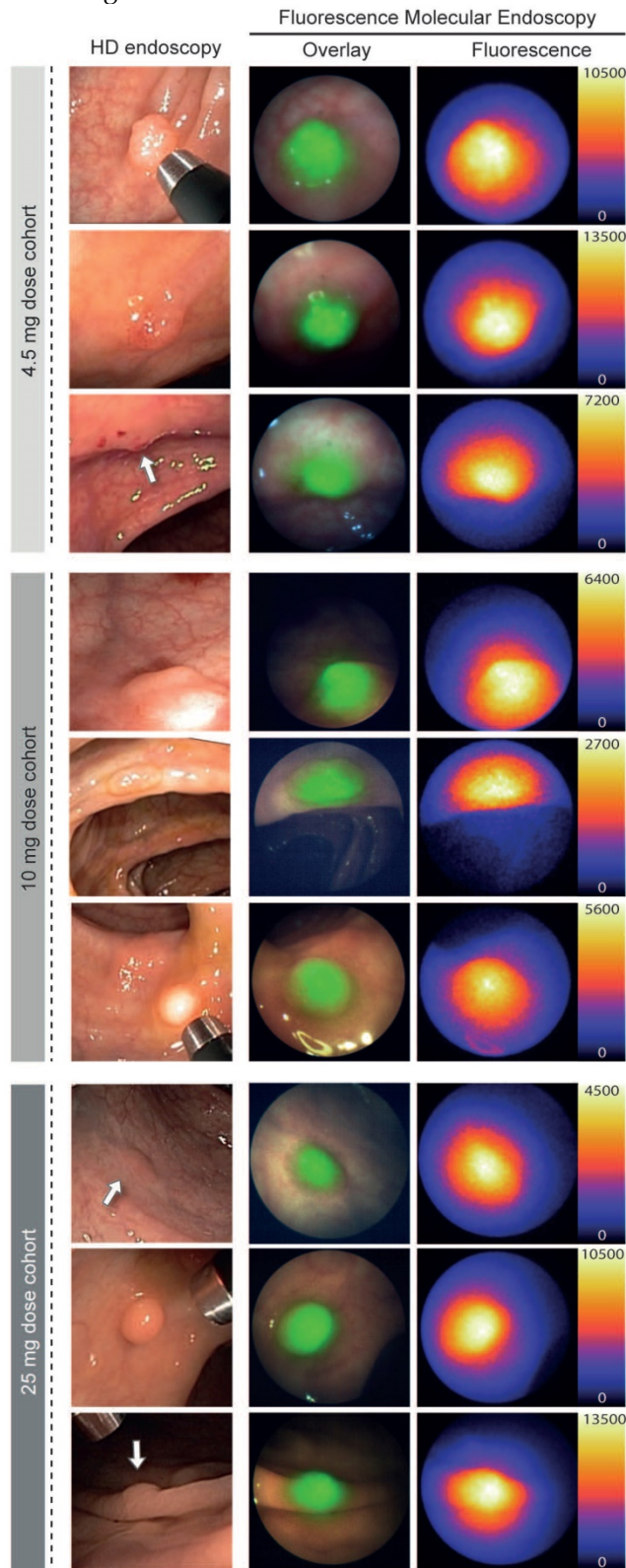
contrast for direct *in vivo* detection at video rate (10 frames per second [FPS]). Small adenomas (<3 mm) were clearly fluorescent in the 25 mg bevacizumab-800CW dose cohort enabling real-time visualization of all adenomas at video rate (Figure 3 and Video S1 and S2), though the fluorescence signal varied in the 4.5 mg bevacizumab-800CW dose cohort hampering real-time detection in this lowest dosing cohort. Moreover, we were even able to detect small adenomas (<3 mm) present in the background of bright fluorescent adenomas (Figure 3 - second row). Normal colorectal mucosa showed only minimal fluorescence, resulting in a clear delineation of the fluorescent adenomas. We did not observe false positives in this feasibility study since normal-appearing colorectal tissue during HD inspection showed no significant NIR fluorescence with NIR-FME. In a few cases, where bowel preparation was insufficient, we did observe a detectable amount of NIR fluorescence in remaining feces. This NIR fluorescence is probably due to the native fluorescence present in fecal remnants, most likely originating from unrefined chlorophyll-containing ingredients like spinach [15].

#### MDSFR/SFF spectroscopy: fluorescent signal quantification

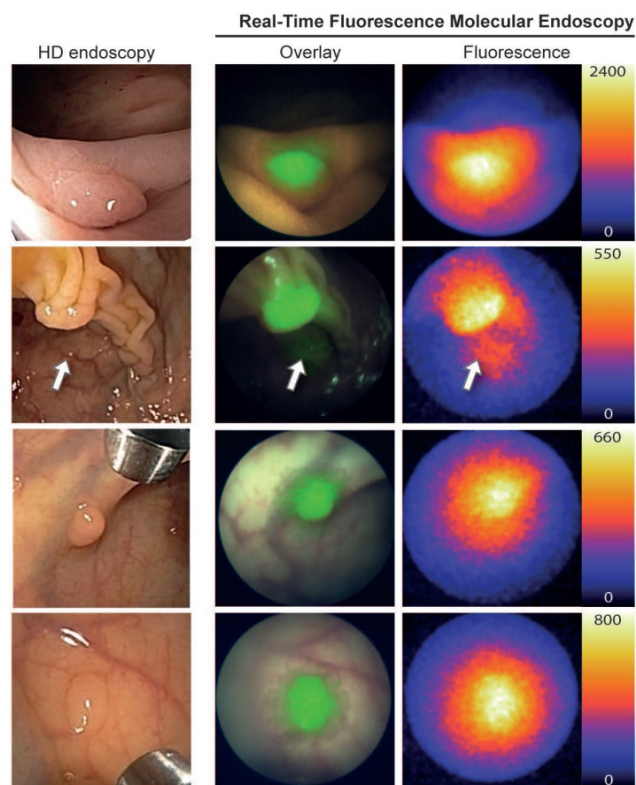
In total, the intrinsic fluorescence intensities were determined of 39 adenomas and 27 normal colon biopsies. This revealed a median adenoma-to-normal ratio for the 25 mg dose cohort of 1.84. There was a 40% increase of intrinsic fluorescence of adenomas for the 25 mg cohort compared to the 10 mg cohort (Figure 4). In contrast, the intrinsic fluorescence intensities of normal tissue remained constant for all dose cohorts. The correction factor to correct the raw fluorescence for tissue optical properties ranged between 1.65 and 3.57. Tissue absorption, mainly by hemoglobin, was the main actor in this, while differences in scattering made a smaller but still significant contribution. The resulting intrinsic fluorescence spectra resembled the emission spectrum of bevacizumab-800CW in PBS, which confirms that the measured fluorescent signals are tracer derived (Figure S2).

Based on the intrinsic fluorescence determined with MDSFR/SFF spectroscopy, an estimation could be made of the tracer concentration present in the tissue. This showed a median bevacizumab-800CW concentration 4.81 nmol/mL in the 10 mg adenomas, compared to 6.86 nmol/mL in the 25 mg adenomas. The median tracer concentration in normal tissue was 3.82 nmol/mL (10 mg cohort) vs 3.73 nmol/mL (25 mg cohort). These quantified measurements confirm our *in vivo* NIR-FME results, in which we observed

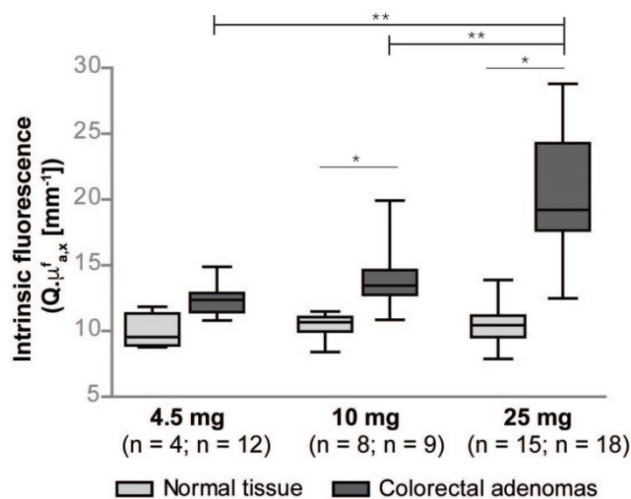
improved fluorescence visualization of adenomas in the 25 mg dose cohort.



**Figure 2. Wide-field VEGFA-targeted fluorescence endoscopy.** Three adenomas per tracer-dose cohort. The clinical white-light images gained with a HD video endoscope (first column), combined with representative overlay images (second column) and NIR fluorescence images (third column) gained with the NIR fiber bundle. The overlay images are automatically generated by the software, showing the highest fluorescence intensities in bright green and the very low fluorescence intensities as absent. The fluorescence images were taken with different exposure times.



**Figure 3. Real-time *in vivo* NIR-FME images.** Adenomas from the 25 mg dose cohort demonstrating the ability of the NIR-FME system to visualize small and flat adenomas at video frame rate (10 frames per second). The white arrow indicates a second small adenoma. The overlay images are automatically generated by the software, showing the highest fluorescence intensities in bright green and the very low fluorescence intensities as absent.



**Figure 4. Fluorescence quantification by MDSFR/SFF spectroscopy.** Box plot (median, 10-90 percentile) showing the intrinsic fluorescence ( $Q_i^{a,x}$  [mm<sup>-1</sup>]) per bevacizumab-800CW tracer-dose cohort for both LGD containing adenomas and the normal colorectal tissue (biopsies). For all dose cohorts, a significant difference in fluorescence intensity can be observed between the benign and premalignant tissue, which increases with increasing tracer dosages. Note that the fluorescence in the normal tissue stays constant in the 10 and 25 mg cohorts, regardless of the tracer dose used. The median adenoma-to-normal ratio of intrinsic fluorescence was 1.84 for the 25 mg cohort. \* =  $P < 0.05$ ; \*\* =  $P < 0.001$ .

**Ex vivo tissue analyses: fluorescent signal qualification.**

In total, 49 FFPE adenomas (4.5 mg n = 21; 10 mg

n = 11; 25 mg n = 17) and 24 normal tissue biopsies (4.5 mg n = 8; 10 mg n = 4; 25 mg n = 12) were analyzed. Of the 49 FFPE adenomas, 48 contained LGD and one showed HGD. Within the adenomas, the tracer was mainly localized in the dysplastic areas compared to the normal tissue within the adenoma section (Figure 5A). NIR fluorescence was localized between the adenomatous crypts, within the stromal tissue (Figure 5B). Normal colorectal tissue within the sections of adenomas and in the normal mucosa biopsies both showed negligible NIR fluorescence (Figure 5C).

**Target-validation: VEGFA immunohistochemistry**

We observed a clear difference in VEGFA expression levels between the dysplastic crypts and the normal colon crypts (Figure 6). All adenoma samples expressed VEGFA, of which 96% showed a high staining intensity and 4% an intermediate staining (mean H-score: 286). In contrast, the normal colon tissue showed a lower mean intermediate H-score, namely 123 for normal crypts within the adenoma sections, versus 174 for biopsies of normal mucosa.

**Discussion**

In this study, we demonstrate that colorectal adenomas can be identified with VEGFA-targeted NIR-FME with concurrent high-definition white-light endoscopy. Based on the real-time observations in FAP patients, we identified 25 mg bevacizumab-800CW as the best-performing tracer dose. With this dose, we were able to identify all small (<5 mm) adenomas at video-rate, even if those were situated in the background of the image, behind a larger fluorescent adenoma. Detailed *ex vivo* fluorescent signal analyses with MDSFR/SFF spectroscopy and microscopy confirmed the specificity of the obtained results. As such, we believe that VEGFA-targeted NIR-FME has the potential to improve real-time, wide-field colorectal adenoma identification. With this first feasibility and dose-finding study, the way is cleared to start a study in high risk patients, to objectify its ‘red-flag’ ability in identifying and improving detection of flat and depressed adenomas.

Previously described targeted FME approaches have been mainly restricted to preclinical application; they described the potential of a wide range of molecular targets, fluorescent labels and administration routes, as well as the use of both wide-field macroscopic and microscopic imaging systems [16,17]. Clinically, different approaches have been tried to obtain wide-field FME for colorectal adenoma detection. Mayinger showed in a feasibility study improved detection of small adenomas after

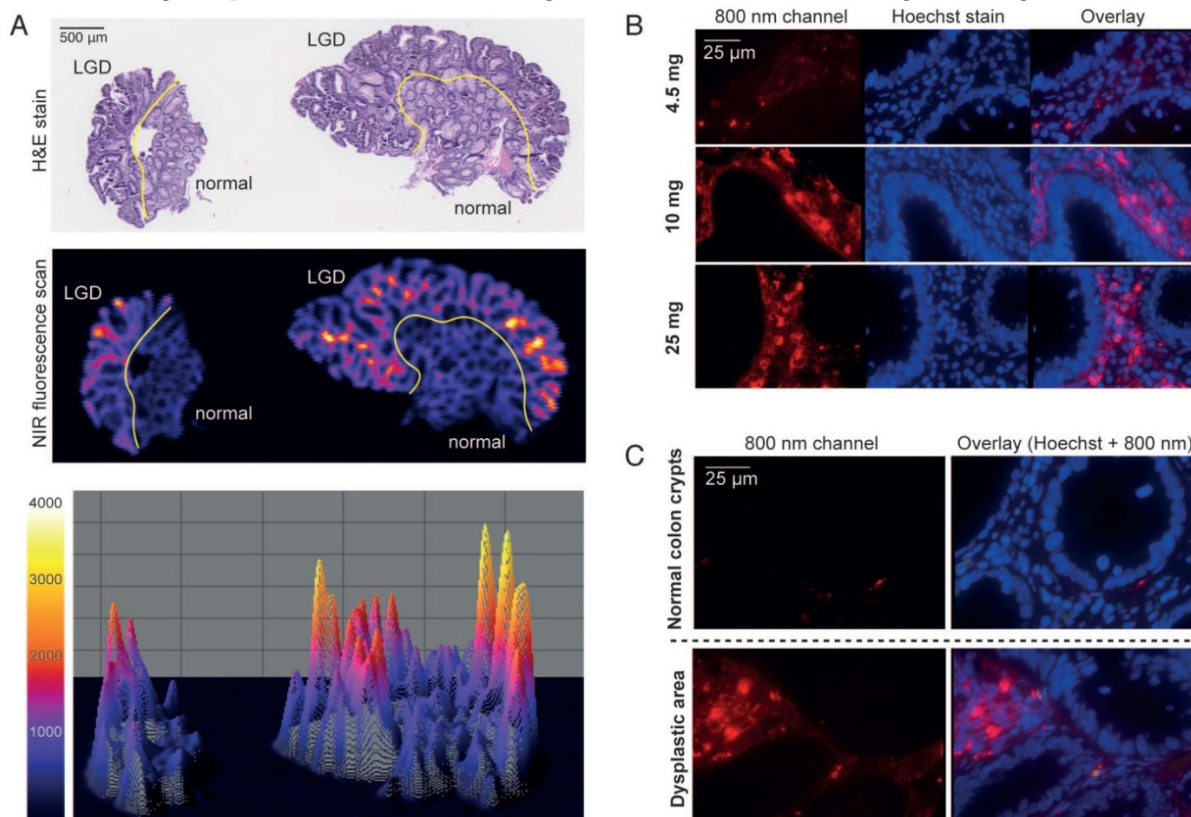


topical application of Hexaminolevulinat (HAL). Since HAL absorption time is around 60 min, tracer administration is challenging. In this study the tracer was administered via an enema which might hamper detection of right-sided lesions [18]. A fluorescent tracer targeting cMET showed promising positive results, though fluorescence imaging was compared with outdated fiber white-light detection which does therefore not reflect current clinical practice [7]. Joshi *et al.* nicely demonstrated specific binding, using *ex vivo* quantification and correction, of a small fluorescent peptide after topical administration on SSA/Ps in the proximal colon identified with white-light HD endoscopy [19]. Future studies applying the tracer before HD identification of the lesions should elucidate the real-time identification potential of promising tracers as “red-flag” technique.

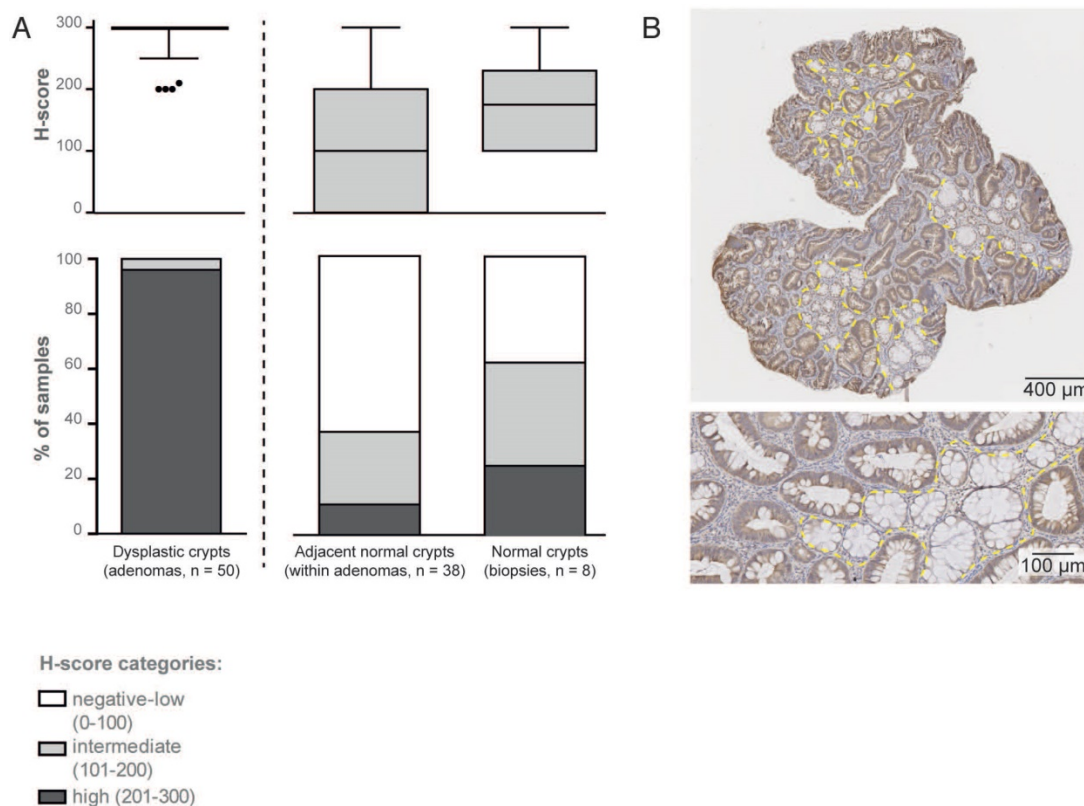
Our FME approach is distinctive from former approaches since we used a tracer in the NIR range to optimally provide *instant* red-flag identification of colorectal lesions. The fluorescence and white-light images could concurrently be captured, as bevacizumab-800CW emits fluorescent light outside of the visible light spectrum; in the NIR light

spectrum. Hence, the fluorescent signals could instantly be superimposed on the white-light images shown to the endoscopist. The clear discrimination between colorectal adenomas and normal tissue is a result of the accumulation of the IV injected bevacizumab-800CW specifically in colorectal adenomas, and the negligible attribution of human autofluorescence in the NIR light spectrum. Future software improvements could automatically alert the endoscopist and thereby even reduce the human factor which might improve adenoma detection rates.

Most likely the optimal tracer dose used during NIR-FME will have to be determined per tracer, organ of interest and indication. The aim of this study was to identify the best dose of IV bevacizumab-800CW for colorectal adenoma identification purposes. In cancer lesions it is thought that, due to the enhanced permeability and retention (EPR) effect, antibodies will accumulate easily, even when only a microdose of tracer is used [20]. However, in case of precancerous lesions, such as adenomatous polyps in which vascular hyperpermeability is not yet present, a higher tracer dose may be required to ensure sufficient fluorescent signal strength.



**Figure 5. Ex vivo fluorescent signal analyses.** (A) Representative NIR fluorescence flatbed scan of a fluorescent adenoma (10 mg dose cohort), containing both dysplasia and normal colon crypts in the same section (HE staining). The fluorescence scan and interactive surface plot demonstrate that the fluorescence intensities are the highest at the sites of dysplasia, a phenomenon that was observed in all three tracer-dose cohorts. (B) Three representative fluorescence microscopy images, demonstrating an observable difference in fluorescence intensities between the three dose cohorts: the 4.5 mg cohort shows a lower signal in the 800 nm channel, compared to the two higher dose cohorts. Fluorescence microscopy did not show a clear difference between the two highest dose cohorts (10 mg vs 25 mg). The left column represents the fluorescence of the tracer (800 nm channel), the middle column shows Hoechst staining of the nuclei and third column displays an overlay of the previous two channels. (C) Representative microscopy images of one adenoma of the 25 mg dose cohort, showing a clear difference in NIR fluorescent signal between areas containing dysplasia and areas containing normal colon crypts; the areas can be distinguished based on the appearance of the crypts, since stacking of the nuclei is typical for dysplasia.



**Figure 6. VEGFA immunohistochemistry results.** (A) Box plot (median, 10-90 percentile) and bar graph, both presenting VEGFA IHC results (H-score) of adenomatous colorectal polyps (LGD and normal crypts) and normal colorectal biopsies; a clear difference in H-score can be observed between the adenomatous crypts and the normal surrounding tissue and normal biopsies. (B) Representative images illustrating the clear difference in VEGFA staining intensities (brown) between dysplastic and normal crypts (areas within dashed yellow lines display normal crypts).

To identify the optimal dose, adequate fluorescent signal quantification is essential since it validates the observed *in vivo* fluorescent signals. The background tissue optical properties, i.e., absorption and scattering, make quantitative measurements on *in vivo* NIR-FME images complicated. MDSFR/SFF spectroscopy can correct raw fluorescent signals for the influence of the local tissue optical properties and was previously validated *in silico* and in optical phantoms, and has been used in *in vivo* pre-clinical models [21-24]. The spectroscopy results showed that an adenoma-to-normal tracer concentration ratio of 1.84 was sufficient to gain real-time visualization of adenomas (25 mg dose cohort). As this ratio is based on actual quantification using spectroscopy, it is not comparable to the usual adenoma-to-normal ratios given in other studies, only obtained by comparing contrast in reflectance images, as is signified by the clear contrast in our *in vivo* images. The applied bevacizumab dose of 25 mg is still far below the conventional therapeutic dose of 5-10 mg/kg and did not show any side-effects [11]. In addition, we subsequently calculated the actual bevacizumab-800CW concentration in the tissue with the assumption that the *in silico* measured fluorescence quantum yield and extinction coefficient of

bevacizumab-800CW are representative for the *in vivo* conditions.

Although our results are promising, our proof-of-principle study has some limitations. First, as the lesions were concurrently detected by white-light endoscopy and NIR-FME, this study cannot address the effect of NIR-FME on improving the adenoma detection rate. Secondly, FAP patients are not an ideal patient population for evaluating adenoma detection miss rates, since they are known to have multiple areas with aberrant crypt foci, and most patients have an ileorectal anastomosis. Nevertheless, we deliberately performed this proof-of-principle study in FAP patients to ensure sufficient adenoma numbers to identify the optimal tracer dose, despite the limited number of available patients. Twenty-five mg bevacizumab-800CW may well be universally used to detect the important SSA/Ps and high-risk Lynch lesions, since we previously showed 94% overexpression of VEGFA in sporadic adenomas (with similar etiology as adenomas of FAP patients), and a comparable 95% in SSA/Ps and 79-96% overexpression in LS patients [8].

In conclusion, this study demonstrates that VEGFA-targeted NIR-FME is a promising optical molecular red-flag imaging approach for colorectal



adenoma detection: our novel probe-based NIR-FME approach meets the current clinical standards since it provides real-time molecular guidance, and thus functional information, without interfering with the regular HD morphological information of white-light endoscopy. Following IV administration of 25 mg of bevacizumab-800CW, NIR-FME highlighted very small dysplastic adenomas with high *in vivo* fluorescent contrast at video-rate, which was confirmed by MDSFR/SFF spectroscopy. Future studies are required to determine the effect of NIR-FME using 25 mg of bevacizumab-800CW on improving the adenoma detection rate of flat and depressed adenomas compared to white-light endoscopy and the thereby the clinical benefit for patients at high risk of developing colorectal cancer, for example patients with Lynch syndrome.

## Abbreviations

CRC: colorectal cancer; EPR: enhanced permeability and retention effect; FAP: familial adenomatous polyposis; FFPE: formalin-fixed and paraffin-embedded; FPS: frames per second; HD: high-definition; H&E: hematoxylin and eosin; HGD: high-grade dysplasia; IHC: immunohistochemistry; IV: intravenous; LGD: low-grade dysplasia; LS: Lynch syndrome; MDSFR/SFF: multi-diameter single fiber reflectance and single fiber fluorescence; *MUTYH*: *MutY* human homolog gene; FME: fluorescence molecular endoscopy; MFI: mean fluorescence intensity; NIR: near-infrared; SSA/P: sessile serrated adenoma / polyp; VEGFA: vascular endothelial growth factor A; WLE: white-light endoscopy.

## Supplementary Material

Supplementary material describes the production and administration of bevacizumab-800CW, the technical details of the NIR-FME system and the MDSFR/SFF spectroscopy device, the determination of the intrinsic fluorescence and the immunohistochemical analysis of VEGFA expression.

<http://www.thno.org/v08p1458s1.pdf>

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## Competing Interests

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