Fluorescently labelled monoclonal antibodies for real-time molecular imaging

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CHAPTER 6

Quantitative fluorescence endoscopy: an innovative endoscopy approach to evaluate neoadjuvant treatment response in locally advanced rectal cancer

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ABSTRACT

Quantitative fluorescence endoscopy (QFE) is a new technique that can visualise and quantify fluorescently tagged tumour tissue. In 25 patients with locally advanced rectal cancer (LARC), we evaluated QFE targeting vascular endothelial growth factor A (VEGFA) to detect residual tumour after neoadjuvant chemoradiotherapy (nCRT). QFE detected significantly higher fluorescence in tumour compared with normal rectal tissue and fibrosis, and improved prediction of final pathology results in 16% of patients compared with standard MRI and white light endoscopy. QFE is a promising technique to aid clinical response assessment in patients with LARC and warrants further validation in larger clinical trials.

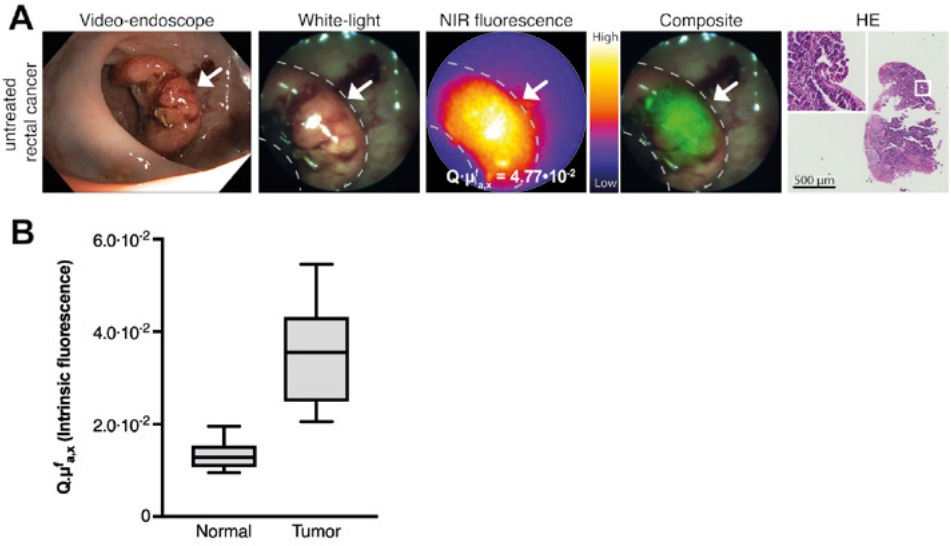


Figure 1 (A) Representative fluorescence images of the quantitative fluorescence endoscopy (QFE) procedure in untreated rectal cancer. From left to right: a high-definition white-light video endoscope image; a white-light image from the QFE fibreoptic, followed by the corresponding near-infrared (NIR) fluorescence image captured with an exposure time of 100 ms and the composite image of both modalities. The maximum quantified fluorescence value is depicted on the NIR fluorescence image. The rightmost image shows the HE staining of a forceps biopsy of the fluorescent area, confirming adenocarcinoma. (B) Fluorescence quantification results in 10 untreated patients. Tumour tissue shows higher fluorescence compared with normal rectal tissue. Boxplot centreline is at median, the bounds of the box at 25th to 75th percentiles, the whiskers depict the minimum–maximum

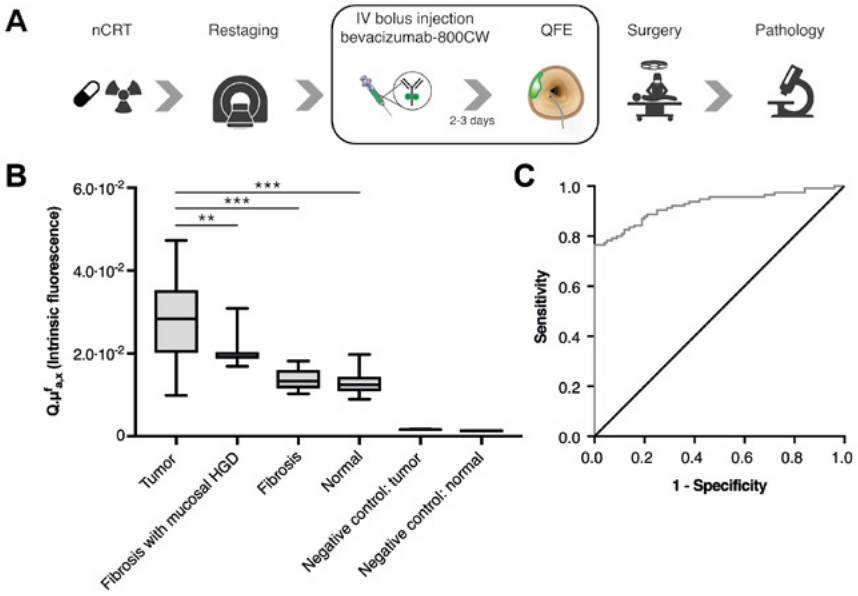
RESULTS

Patients with LARC receive nCRT followed by surgery to achieve local disease control. Interestingly, 15%–27% of patients have a pathological complete response, that is, no residual cancer cells are found in the surgical specimen. (1–3) There is an increasing interest in identifying patients with a clinical complete response before surgery, as non-operative management for these patients is associated with high survival rates, reduced morbidity and improved functional outcomes. (4–8) However, assessing tumour response after nCRT is challenging. White-light endoscopy provides only morphological information, while MRI cannot always distinguish viable tumour from fibrosis. (9–11) QFE is a novel endoscopy technique that visualises and quantitatively measures the presence of targeted fluorescence tracers in tissue. We hypothesised that VEGFA-targeted QFE can be of additional value in restaging patients with LARC. In untreated patients, QFE showed clearly enhanced fluorescence in all rectal tumours compared with normal rectal tissue (figure 1A). The tumour-to-normal ratio of 3.1 (figure 1B) signifies QFE can be used to localise rectal cancer. In this pilot study, we included 25 patients with LARC who were treated with nCRT (online supplementary table S1). QFE was performed at day of surgery, which enables comparison of QFE with standard clinical restaging (MRI and white-light endoscopy) and correlation to histopathology of the surgical specimen (figure 2A).

In all patients, vital tumour tissue showed high fluorescence compared with normal rectal tissue or fibrosis (online supplementary figure S1). Fluorescence quantification confirmed that fluorescence of tumour tissue (n=155 measurements) was higher than normal rectal tissue and fibrosis (n=100 measurements) ($p<0.001$) (figure 2B). The receiver operating characteristic curve showed a fluorescence cut-off value of 2.00×10^{-2} (area under the curve 0.925) (figure 2C,D). QFE was true positive in 21 of 25 patients as mucosal tumour (n=19, figure 3A) or even submucosal tumour (n=2, figure 3B) was confirmed by histology. QFE was truly negative in 2 of 25 patients, as histology confirmed pathological complete response (ypT0N0) (figure 3C). QFE was false positive in 1 of 25 patients, who showed extensive polypoid tissue on white-light endoscopy with one apparent fluorescent spot, where histology showed no invasive tumour (ypT0N0), but instead one locus with high-grade dysplasia (figure 3D). In 1 of 25 patients, QFE was false negative, and histology showed microscopic residual tumour: one locus situated in the submucosa.

Compared with standard clinical restaging, QFE would have changed the diagnosis in 4 of 25 patients (16%) (online supplementary figure S2). Three patients, categorised as clinical complete responders by MRI and white-light endoscopy, showed fluorescence with QFE and indeed showed vital tumour at histopathological examination (n=2) or regrowth already after 2 months of watchful waiting (n=1). One patient was clinically categorised as having residual tumour, but QFE showed low fluorescence and pathological examination confirmed a pathological complete response.

In our small sample size of 25 patients, the initial positive predictive value was 95% for QFE compared with 87.5% for MRI and 90% for white-light endoscopy. The accuracy of QFE was 92% compared with 84% for MRI and 80% for white-light endoscopy.



D

	Pathological residual tumor	Pathological complete response	Total
QFE positive $Q{\cdot}\mu'_{a,x} \geq 2.00{\cdot}10^{-2}$	21	1*	22
QFE negative $Q{\cdot}\mu'_{a,x} < 2.00{\cdot}10^{-2}$	1	2	3
Total	22	3	25

* High-grade dysplasia in surgical specimen

Figure 2 (A) Schematic overview of the clinical and study procedures. 4.5 mg bevacizumab-800CW was intravenously administered 2–3 days prior to quantitative fluorescence endoscopy (QFE). QFE consisted of wide-field fluorescence imaging, followed by fluorescence quantification using MDSFR/SFF spectroscopy and taking four forceps biopsies of normal rectal tissue (10 cm proximal from the tumour) and of 4 areas of the rectal tumour when present. (B) Fluorescence quantification results of the QFE procedures after neoadjuvant chemoradiotherapy (nCRT), depicted per tissue type. Tumour tissue shows higher fluorescence compared with fibrosis and normal tissue. Negative control tissue (of measurements of tumour and normal rectal tissue from a patient without tracer) showed no detectable fluorescence, signifying the measured fluorescence originated from the tracer. Boxplot centreline is at median, the bounds of the box at 25th to 75th percentiles, the whiskers depict the minimum–maximum. **p≤0.01; ***p≤0.001, one-way ANOVA test with Tukey post hoc analysis. (C) The receiver operating characteristic curve of quantified fluorescence of normal rectal tissue (n=100 measurements) vs tumour tissue (n=115 measurements) shows an area under the curve of 0.925. Normal rectal tissue included normal rectal tissue measurements of all patients and fibrosis measurements of pathological complete response. Tumour tissue included all lesion measurements of all patients with residual tumour at pathological examination. (D) Contingency table. HGD, high-grade dysplasia.

COMMENTS

This is the first-in-human study demonstrating that *in vivo* VEGFA-targeted QFE can improve the response assessment of patients with LARC after nCRT. We observed a sensitivity of 95% and accuracy of 92% for QFE compared with the reported respectively 71% and 89% of MRI combined with white light endoscopy. (10) The addition of QFE to MRI and white light endoscopy resulted in more accurate clinical restaging in 16% of patients. Moreover, QFE is easy to perform during white-light endoscopy: the imaging and spectroscopy probes can be inserted in the working channel of any clinical video endoscope, the QFE measurements are operator independent and the procedure takes slightly more time (5–10 min). Importantly, no tracer-related or procedure-related adverse events were observed in this study.

When QFE is applied for restaging purposes, fluorescence quantification is important. Wide-field fluorescence visualisation alone does not necessarily reflect true tracer accumulation as fluorescence is influenced by tissue optical properties and could therefore lead to incorrect recommendations in clinical practice. By quantifying the fluorescence with multi-diameter single fiber reflection/single fiber fluorescence (MDSFR/SFF) spectroscopy, the fluorescence signals are corrected for tissue optical properties like scattering and absorption, circumventing this problem. (12,13)

Recent follow-up data showed that 19% of patients in watchful waiting, experience early tumour regrowth within 12 months. (14) The majority of these patients had ypT3 or ypT4 disease at salvage, suggesting the presence of residual disease, intraluminal and also in deeper layers of the rectum. QFE might improve identification of these patients, as in this study QFE measured increased fluorescence in two of three patients with only submucosal tumour, that is, no tumour reaching the rectal mucosa. We hypothesise that bevacizumab-800CW could accumulate at the mucosal side because the tumour microenvironment was not yet normalised after nCRT, with still increased levels of VEGFA. A tracer that accumulates in the microenvironment could therefore offer an advantage for restaging compared with tracers that target proteins on tumour cell membranes. In addition, bevacizumab-800CW is a near infrared tracer allowing deeper tissue penetration compared with tracers in the visible spectrum.

In this pilot study, QFE was false positive in one patient who turned out to have one locus of high-grade dysplasia at the rectal lumen. This is not surprising as a former study showed that bevacizumab-800CW also accumulates in low-grade and high-grade dysplastic adenomas which hampers discrimination between dysplasia and cancer. (13) QFE was false negative in one patient who had one microscopic tumour locus present in the submucosa. Possibly, raising the tracer dose could provide stronger fluorescence signals and thus improve QFE detection. A clinical dose-finding study using bevacizumab-800CW for detection of colorectal adenomas reported that a higher tracer dose of 25 mg increased the target-to-background ratio almost twofold. (13)

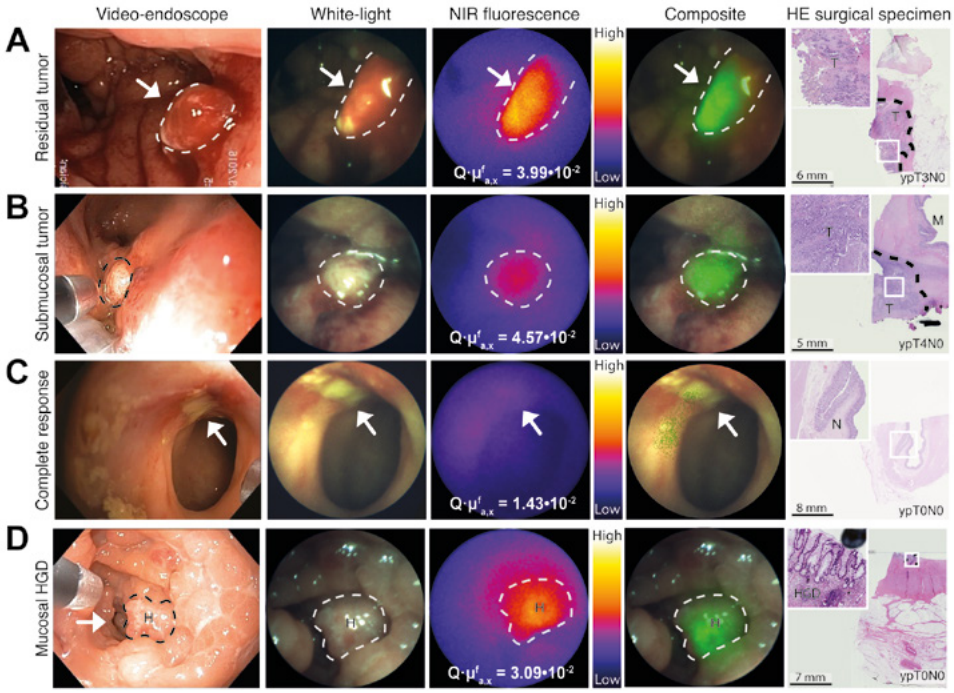


Figure 3 (A–D) Representative images of the quantitative fluorescence endoscopy (QFE) procedure after neoadjuvant chemoradiotherapy of a patient with (A). residual tumour, (B) submucosal tumour, (C) mucosal high-grade dysplasia (HGD) and (D) a pathological complete response. From left to right: a high-definition white-light video endoscope image of the rectal tumour; a white-light image from the QFE fibreoptic, followed by the corresponding near-infrared (NIR) fluorescence image captured with an exposure time of 100 ms and the composite image of both modalities. The maximum quantified fluorescence value is depicted on the NIR fluorescence image. The rightmost column depicts an HE staining of the surgical specimen in which the pathological TNM stage is indicated.

Potentially, future complementary detection systems such as optoacoustic imaging, which combines the rich contrast of optical imaging with the higher penetration of radiofrequency waves, may further improve submucosal evaluation.

Our study has some limitations. We found a relatively low specificity and negative predictive value of QFE (67%) in this feasibility study, which might be due to the relatively small sample size. Next to this, the included patients were referred to our tertiary centre and represent patients with relatively complex LARC with extensive tumour (T4 in 40%) and high nodal stage (N2 in 64%) compared with the patients with relatively uncomplex LARC in standard practice. This also resulted in a relatively small portion of patients who experienced a pathological complete response (12%), compared with 15%–27% pathological complete response described in the literature.(1,2)

In conclusion, the results of this pilot study, even in this small group of patients, are encouraging and are potentially a first step towards quantitative fluorescence endoscopy for tumour response evaluation following neoadjuvant treatment. Ultimately, the combination of MRI, white-light endoscopy and QFE may prove to be the strategy to evaluate individual patient response and guide clinical decision making. To realise this strategy, the capability of QFE in clinical response evaluation in patients with LARC, including determination of a definitive cut-off value that discriminates tumour from normal tissue, needs further evaluation in a larger prospective cohort.

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SUPPLEMENTARY MATERIAL

Summary of the study design

Patients with LARC were given a single dose of bevacizumab-800CW intravenously 2-3 days prior to the procedure. QFE was performed after the completion of nCRT, at day of surgery. In ten patients, QFE was also performed in untreated patients. Tracer uptake in LARC was compared to normal tissue in order to evaluate the detection of residual tumour and aid clinical response assessment after completion of nCRT. Patients also underwent conventional clinical restaging (MRI and white-light endoscopy). All results were compared to the gold standard: histopathological staging of the surgical specimen.

Study population

A total of 25 patients with proven LARC were enrolled between October 2013 and December 2016, in this non-blinded, prospective, single centre feasibility study. Patients were required to have histopathologically confirmed adenocarcinoma, with the lower margin within 16 cm from the anal verge. The pelvic MRI indicated at least one of the following criteria: cT4a, cT4b, N2, presence of tumour cells in the vasculature beyond the muscularis propria –extramural venous invasion (EMVI)–, presence of tumour or lymph node <1 mm from the mesorectal fascia (MRF) or positive lateral lymph nodes. Patients were eligible only if the multidisciplinary team decided on long-course nCRT. Patients were allowed also to be included in the RAPIDO trial (ClinicalTrials.gov Identifier NCT01558921). Key exclusion criteria were concurrent uncontrolled medical conditions and pregnancy or breast-feeding. Eligible patients were identified during the multidisciplinary colorectal cancer meeting at the University Medical Center Groningen (UMCG, Groningen, the Netherlands). All patients gave written informed consent for participation in the study before inclusion. The study protocol was approved by the Medical Ethics Committee of the UMCG and registered with ClinicalTrials.gov (NCT01972373).

Patient and Public Involvement

The study was supported by the Dutch Cancer Society. There was no patient involvement in study design, interpretation of results or writing of the manuscript.

Clinical procedures

Neoadjuvant treatment

Patients underwent nCRT before surgery, consisting of 28 doses of 1.8 Gy and oral capecitabine (825 mg/m² twice daily orally during radiotherapy course or 1000 mg/m² twice daily during 2 cycles of 14 days during radiotherapy course) or according to the study arm of the RAPIDO trial (n=2): 5 doses of 5 Gy, followed by 6 courses every 3 weeks of oxaliplatin intravenously (130 mg/m²) at day 1 and oral capecitabine (1000 mg/m²) twice daily for 14 days starting at day 1 of the course. Dose adjustments were made in the event of side effects. No post-operative chemotherapy was administered, in line with our national guideline. (1)

Clinical restaging

All patients underwent radiological restaging after nCRT, which consisted of a computer tomography (CT) scan of chest and abdomen and a diffusion-weighted MRI scan of the pelvis. Tumour (T), lymph node (N) and metastasis (M) stage were assessed, together with EMVI and MRF, according to the TNM classification off the American Joint Committee on Cancer (5th edition). Endoscopic ultrasound was not used as standard clinical restaging modality.

Surgical resection

After the restaging CT and MRI, the surgical plan was formulated. Surgery consisted of abdominoperineal resection, low anterior resection or a more extended procedure like partial or full pelvic exenteration in order to reach a tumour-free circumferential resection margin. Although watchful waiting was not part of standard clinical care in our institution at the moment of the study, two patients requested this even though MRI and white-light endoscopy were inconclusive.

Pathological examination

Standard pathologic tumour staging of the resected specimen was performed by dedicated gastrointestinal cancer pathologist blinded for QFE results. The pathologic stage (ypTN) was recorded according to the fifth edition of the TNM classification, the clinical standard for the Netherlands. Circumferential resection margin involvement and lymphovascular invasion status were documented. pCR was defined as absence of viable adenocarcinoma cells in the surgical specimen (ypT0N0).

Study procedures

10 included untreated patients with LARC received a QFE procedure right after diagnosis, before nCRT. This was performed to verify if bevacizumab-800CW would accumulate in the rectal tumour tissue. Twenty-five patients received a QFE procedure after nCRT, at day of surgery, enabling direct correlation with the current clinical standards: radiological restaging (cTNM), white-light video endoscopy and the pathological outcome of the surgical specimen (ypTNM).

Tracer production and administration

The monoclonal antibody bevacizumab (Roche, Hertfordshire, United Kingdom) was labelled under cGMP conditions with the near-infrared fluorophore IRDye800CW (IRDye800CW-NHS ester; LI-COR Biosciences, Lincoln, NE, USA) at the Department of Clinical Pharmacy and Pharmacology of the UMCG. (2) This was originally performed in a 4:1 dye-to-protein molar ratio. After the first six patients, the dye-toprotein molar ratio was changed to 2:1 to improve long term stability. No changes were seen in immunoreactivity tests. Patients received 4.5 mg of bevacizumab-800CW in accordance with

microdosing limits as defined by the FDA. (3) Tracer was administered via intravenous bolus injection, 2 to 3 days prior to the QFE procedure, the optimal time-to-imaging interval based on experience with ^{89}Zr-bevacizumab PET-scans. (4) No tracer-related serious adverse events were reported, in accordance with previous clinical studies. (5–8)

White–light endoscopy procedure

All study subjects first received white-light endoscopy with a routine clinical high definition video endoscope, immediately followed by QFE. Tumour response was endoscopically assessed by a dedicated gastroenterologist (W.B.N.) according to watchful waiting criteria: CR was diagnosed if residual tumour was absent, and only a flat, white scar with or without telangiectasia was present. Potential CR was diagnosed when a small, flat ulcer with smooth edges without signs of residual polypoid tissue was present. Every other type of ulcer or mass was considered as definite residual tumour. (9)

QFE procedure

After high-definition white-light inspection of the rectum with a routine clinical high definition video endoscope, the wide-field optical fibre was inserted through the working channel of the endoscope for wide-field fluorescence imaging. The gastroenterologist observed the presence, distribution and intensity of fluorescence signals in normal rectal tissue and in all rectal lesions present at endoscopy. Fluorescence was visually categorized as low (no difference with surrounding normal rectal tissue), intermediate (elevated, but difficult to clearly differentiate from surrounding normal rectal tissue) or high (clear differentiation from surrounding normal rectal tissue based on fluorescent signals). Images of normal tissue and LARC cancer tissue were digitally recorded with an exposure time of 1 frame per second and at video rate (10 frames per second). Subsequently, the spectroscopy fibre was inserted through the working channel of the endoscope and held onto tissue of interest, to perform *in vivo* point measurements for quantification of the NIR fluorescence. Quantification of minimal 3 different tumour areas and normal rectal mucosa was performed, preferably 10 cm proximal of the rectal tumour. At the end of the QFE procedure, four small forceps biopsies were taken of normal rectal tissue and of every tumour location where quantification was performed. *Ex vivo* spectroscopy measurements were performed on these fresh biopsies to enable direct correlation of NIR fluorescence with histopathology. As there were no significant differences between the *in vivo* and *ex vivo* spectroscopy measurements, the measurements depicted in the result section were grouped. Afterwards, the tissue biopsies were formalin-fixed and paraffin-embedded (FFPE) or snap-frozen in liquid nitrogen and stored at -80° Celsius.

QFE system

Wide-field fluorescence imaging was provided by an imaging platform (SurgVision BV, Groningen, the Netherlands) consisting of an optical fibre-bundle coupled to a charge-coupled digital (EM-CCD) camera, sensitive for NIR light, and a separate camera for colour detection, as described previously. (7,8) Fluorescence excitation was provided by two class IIIb lasers (750 nm); white-light was provided by a LED light source. The wide-field fibre images (colour, fluorescence and composite) were displayed live on a separate monitor for the gastroenterologist.

Fluorescence quantification was performed with a Multi Diameter Single Fiber Reflectance and Single Fiber Fluorescence (MDSFR/SFF) spectroscopy device. The *in vivo* measurements were executed with a fibre-bundle consisting of two concentric rings, the *ex vivo* measurements were performed with a different fibre-bundle consisting of 2 adjacent fibres (0.4 and 0.8 mm). During a measurement, two consecutive reflection spectra were acquired from which the tissue light absorbance and light reflection were calculated. (10–12) This was immediately followed by a fluorescence spectrum measurement. The intrinsic fluorescence (Q.µfa,x) of bevacizumab-800CW was acquired by correcting the fluorescence spectrum for the calculated tissue optical properties. (10,13,14) We calculated the local tracer concentration based on the *in vivo* quantified fluorescence, the molar extinction of the tracer and the fluorescence quantum yield. (8) In 6 patients, all with clear fluorescent endoluminal tumour present, the fluorescence quantification could not be performed due to malfunction of the spectroscopy device (lamp broken). Correlation of QFE findings with radiological and pathological staging

To assess the value of QFE after nCRT, QFE findings were compared to the clinical restaging findings (MRI and high-definition white-light endoscopy) and correlated to the gold standard: pathological staging (ypTNM). The haematoxylin and eosin (HE) staining was performed on 4 µm FFPE tissue sections as standard clinical staining by our Pathology Department.

Statistical methods

Descriptive statistics were generated to describe patient characteristics and the association between QFE and pathological outcome. The intrinsic fluorescence (Q·µfa,x) measurements of different tissue types after nCRT was analysed with a oneway ANOVA test with Tukey post-hoc analysis. A ROC curve was generated from all fluorescence measurements obtained from normal rectal tissue versus tumour tissue. Normal rectal tissue included normal rectal tissue measurements of all patients and fibrosis measurements of pathological complete responders. Tumour tissue included all lesion measurements of all patients with residual tumour at pathological examination. The median and maximum values of the intrinsic fluorescence measurements (Q·µfa,x) were correlated, showing a good correlation ($R^2 = 0.84$, $P<0.0001$, data not shown). P values lower than 0.05 were regarded as statistically significant. IBM SPSS Statistics, version 23.0 (SPSS inc.)

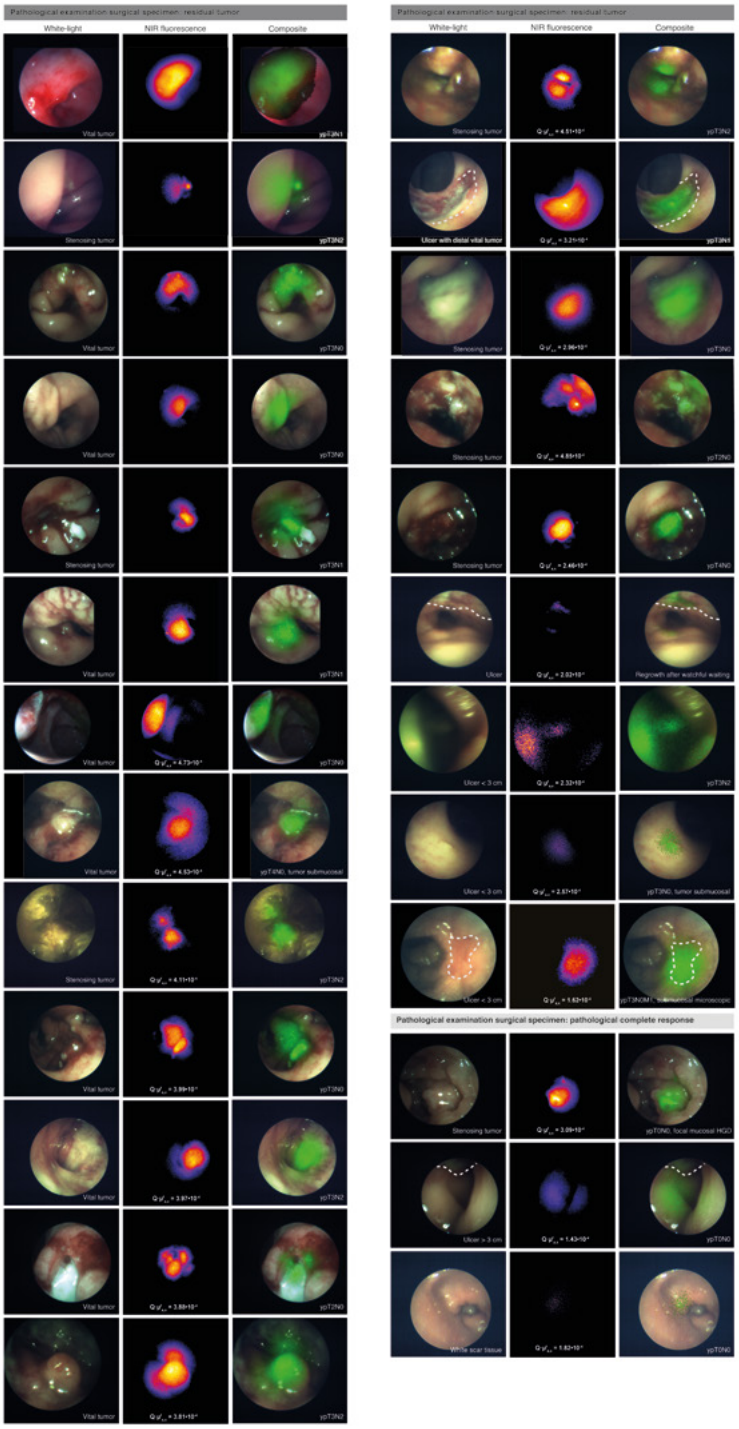
was used for all statistical analyses. All authors had access to the study data and reviewed and approved the final manuscript.

Patient characteristics

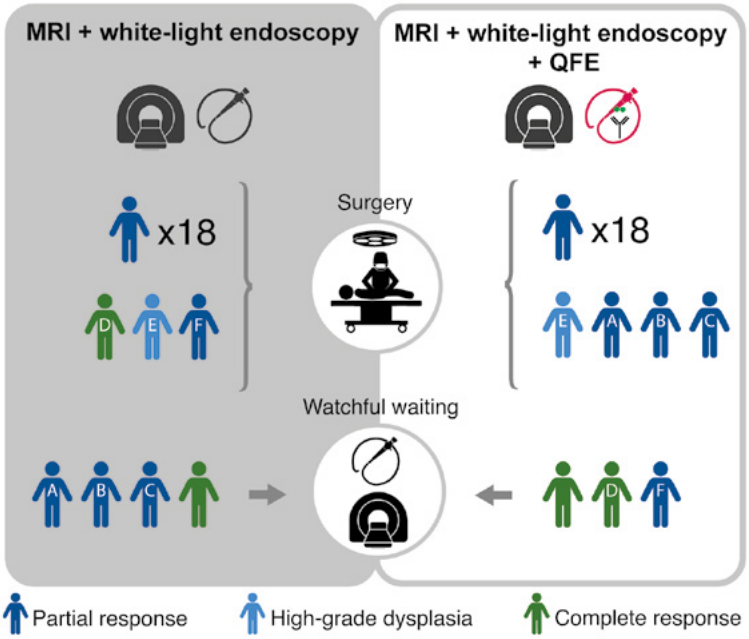
Twenty-five patients diagnosed with LARC were enrolled in the study. Ten of these patients received a baseline QFE prior to nCRT (untreated cancer), and all 25 patients received QFE after nCRT (table 1)

Supplementary table S1 Patient and tumour characteristics

Characteristic	No.	%
Sex	61 (31-76)	
Median age, in years (range)		
Male	15	60%
Female	10	40%
Endoscopic findings at time of diagnosis		
Non-passable stenosis	7	28%
Radiologic staging (MRI pelvis and CT chest+abdomen)		
cT3 N0	2	8%
cT3 N1	5	20%
cT3 N2	10	40%
cT4 N1	4	16%
cT4 N2	4	16%
Neoadjuvant chemoradiotherapy regimen		
Capecitabine 825 mg/m2 bid day 1-28 + 28x 1.8Gy radiotherapy	18	72%
Capecitabine 1000 mg/m2 bid day 1-14 and 25-38 + 25x 2Gy radtiotherapy	5	20%
6 cycles of capecitabine/oxaliplatin + 5x 5Gy radiotherapy	2	8%
Main endoscopic findings at restaging		
Residual tumour/polypoid tissue	19	76%
Ulcer >3 cm	2	8%
Ulcer <3 cm	3	12%
White-scar tissue	1	4%
Type of surgery		
Low anterior resection	14	56%
Abdominoperineal resection	11	44%
Pathological staging		
ypT0N0 (pCR)	3	12%
ypT2N0	4	16%
ypT3N0	6	24%
ypT3N1	3	12%
ypT3N2	6	24%
ypT3N0M1	1	4%
ypT4N0	2	8%


Pathological examination surgical specimen: residual tumor
White-light
NIR fluorescence
Composite
Vital tumor
ypT3N1
Stenosing tumor
ypT3N2
Vital tumor
ypT3N0
Vital tumor
ypT3N0
Stenosing tumor
ypT3N1
Vital tumor
ypT3N1
Vital tumor
ypT3N0
Vital tumor
ypT4N0, tumor submucosal
Stenosing tumor
ypT3N2
Vital tumor
ypT3N0
Vital tumor
ypT3N2
Vital tumor
ypT2N0
Vital tumor
ypT3N2
Pathological examination surgical specimen: residual tumor
White-light
NIR fluorescence
Composite
Stenosing tumor
ypT3N2
Ulcer with distal vital tumor
ypT3N1
Stenosing tumor
ypT3N0
Stenosing tumor
ypT2N0
Stenosing tumor
ypT4N0
Ulcer
Regrowth after watchful waiting
Ulcer < 3 cm
ypT3N2
Ulcer < 3 cm
ypT3N0, tumor submucosal
Ulcer < 3 cm
Pathological examination surgical specimen: pathological complete response
Stenosing tumor
ypT0N0, focal mucosal HGD
Ulcer > 3 cm
ypT0N0
White scar tissue
ypT0N0

Supplementary figure S1 (facing page). One representative, near infrared (NIR) fluorescence and composite fiberoptic image of all 25 quantitative fluorescence endoscopy (QFE) procedures performed after neoadjuvant chemoradiotherapy (nCRT). In the left column, the QFE white-light images are presented together with some additional observational notes. IN the middle column, the QFE NIR fluorescence images are presented, the maximum quantified fluorescence of the lesion is depicted in all cases that were quantified. In the right column, the QFE composite images of the former two columns are depicted. All three images were also visible in real-time at video rate for the gastroenterologist during the endoscopy procedure. The fluorescence images in this figure were acquired with different exposure times and are not scaled to one another. Therefore, visual comparison of fluorescence intensities between different procedures is not possible. Many factors influence the wide-field fluorescence visualization i.e. fibre age, varying distance between lesion and fibre tip and different tissue optical properties.



Supplementary figure S2: Schematic visualization of the potential added value of QFE to MRI and white-light endoscopy as restaging modality after nCRT. Clinical restaging with white-light endoscopy and magnetic resonance imaging (MRI) diagnosed four patients as having a clinical complete response, of which only one patient had a pathological complete response. Twenty-one patients were suspected of having residual tumour, of which two patients had a pathological complete response. However, by combining quantitative fluorescence endoscopy (QFE) findings with these clinical results, restaging would diagnose three patients with complete response, 22 patients suspected of having residual tumours. By adding QFE results, restaging diagnosis might be corrected in four out of 25 patients (16%). In patients A-C, QFE detected clear fluorescence, indicating residual tumour in three patients clinically categorized as as potential complete responders. In patient D, QFE showed low fluorescence , thus recognizing a complete response in a patient categorized by conventional staging methods as having residual tumour. In patient E, QFE showed the only false-positive result, which was also false positive on conventional imaging with suspected residual tumour, but high-grade dysplasia was shown at histological examination. In patient F, QFE was false-negative (i.e. ulcer <3 cm) but the surgical specimen contained only small microscopic submucosal tumour foci.