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Advanced endoscopic imaging of esophageal neoplasia; old looks and new visions

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SUMMARY

Part One: Advanced imaging modalities in perspective

Chapter 2 gives an overview of all current imaging modalities and upcoming techniques in the field of surveillance, work-up and therapy of esophageal adenocarcinoma and squamous cell carcinoma. These modalities include exogenously applied contrast agents, optical filter techniques and functional imaging. Most studies have focussed on surface imaging in Barrett's esophagus, where staining techniques (e.g. *chromoendoscopy*) have not demonstrated a clear additional value over standard white light endoscopy. Optical chromoendoscopy such as narrow band imaging (NBI) may aid in the detection and differentiation of neoplasia, but a clear clinical benefit has not yet been demonstrated. Autofluorescence endoscopy has been shown to increase the targeted detection of early neoplasia, but offers no additional value over high-resolution endoscopy on a patient level. None of these techniques are, therefore, advised for routine endoscopy of Barrett's esophagus.

For long, lugol's staining has been the imaging modality of first choice in esophageal squamous cell neoplasia. Narrow band imaging has important advantages over lugol's staining, and small studies suggest a comparable detection rate. With the current quality of high-resolution white light endoscopy, the endoscopic recognition by endoscopists has become the limiting factor in detecting early neoplasia. Endoscopic training modules are therefore required to increase endoscopists awareness and detection rate.

In **chapter 3** we have focussed on fluorescence imaging: this manuscript gives an overview of current state-of-art of (auto)fluorescence spectroscopy and endoscopy for the imaging of early Barrett's neoplasia. Fluorescence based light-tissue interaction has been successfully designed in point-probe differentiating spectroscopy systems or integrated into wide-field endoscopic systems such as autofluorescence imaging (AFI). A spectroscopy probe, integrated into a regular biopsy forceps, was shown to offer decent discriminatory capabilities, while ensuring spot-on correlation between the measured area and the corresponding histology. With this tool, surveillance endoscopy with random biopsies may become more efficient and sensitive.

AFI was shown to increase the targeted detection of early neoplasia. However, random biopsies could compensate for this effect. The clinical impact of AFI on the diagnosis and treatment of early neoplasia is limited, yet AFI may offer a novel approach in biomarker-based risk-stratification models. Moreover, in combination with new, readily available contrast agents such as fluorescent lectins, fluorescence imaging may receive renewed interest.

After disappointing results with second generation autofluorescence imaging (AFI-II), a third generation AFI system (AFI-III) was developed that was aimed at targeting malignant changes in the cells themselves, rather than secondary structural alterations. In **chapter 4** we described Barrett's patients with endoscopically inconspicuous neoplasia that underwent two diagnostic endoscopies (AFI-II/AFI-III) in a single session. The endpoints of this study were: number of patients and lesions with early neoplasia detected with AFI-II and AFI-III after white-light-endoscopy (WLE) and the value of reinspection of AFI-positive areas WLE and NBI. A total of 45 patients was included (38 males, age 65 years). 19 patients showed high grade intraepithelial neoplasia or intramucosal cancer (HGIN/IMC). AFI-II inspection after WLE increased detection

of HGIN/IMC from 9 to 15 patients (47% to 79%); AFI-III increased detection from 9 to 17 patients (47% to 89%). WLE plus random biopsies diagnosed 13/19 (68%) HGIN/IMC-patients. 104 abnormal AFI-areas were inspected; 23 (22%) showed HGIN/IMC. AFI-II increased detection of HGIN/IMC from 10 to 18 lesions (43 to 78%). AFI-III increased detection from 10 to 20 lesions (43 to 87%). False positive (FP)-rate was 86% for AFI-II and AFI-III. Reinspection with WLE or NBI reduced FP-rate to 21% and 22% respectively, but misclassified HGIN/IMC-lesions as unsuspecting in 54% and 31%, respectively. We therefore concluded that third-generation AFI again showed improved targeted detection of HGIN/IMC in Barrett's esophagus. However, the results do not suggest that AFI-III performs significantly better than conventional AFI-II.

After multiple studies performed on three generations of autofluorescence imaging systems, one important question remained: does AFI impact on the clinical management for patients with early Barrett's neoplasia? In **chapter 5** we have addressed this issue by analyzing the results of 5 previously performed studies on AFI in terms of additional diagnostic and therapeutic value. Therefore, data on patients, endoscopy and histology were extracted from the databases of 5 prospective AFI studies and related to treatment outcome and follow-up. The endpoints were: the diagnostic value of AFI (proportion of surveillance patients with HGIN/IMC detected by AFI-targeted biopsies only), and the therapeutic value of AFI: proportion of patients with any HGIN/IMC lesion detected with AFI that changed initial therapeutic plans based on white-light endoscopy (WLE) or random biopsies (RBx). In the 5 studies, a total of 371 Barrett's patients (mean age 65 yrs, 305 males), referred for surveillance (211) or work-up for early neoplasia (160), were enrolled. HGIN/IMC was diagnosed in 147 patients. Of the 211 patients undergoing surveillance, 39 showed HGIN/IMC: WLE detected 23, RBx detected 11; AFI detected 5 patients. The diagnostic value of AFI in the surveillance – neoplasia naive – population was 5/211 (2%). In 24/371 patients, HGIN/IMC was diagnosed with AFI only. In 33/371 patients, AFI detected additional HGIN/IMC apart from primary WLE/RBx detected lesions. In these 57 patients, the AFI lesions were treated: 26 patients underwent radiofrequency ablation and showed full remission of neoplasia; 31 underwent endoscopic resection of the AFI lesions, showing IMC in 6 cases, that would have otherwise been left untreated, or treated with RFA. In current guidelines, RFA is not indicated for the treatment of IMC. The therapeutic value of AFI therefore was 6/371 (2%). We concluded that, given the low clinical impact of AFI-detected lesions on the diagnosis of early neoplasia and therapeutic decision-making, the role of AFI in routine BE surveillance and work-up is limited. With all results on AFI in hand, we discourage the use of AFI for routine endoscopy of Barrett's esophagus.

In **chapter 6** we subsequently reviewed the clinical relevance of the most widely used commercially available endoscopic imaging techniques with regard to the diagnosis and treatment of early Barrett's neoplasia. We argue that work-up and treatment of early Barrett's neoplasia should be centralised in tertiary referral centers. In this setting, the prevalence of early neoplasia is much higher and procedures are performed under optimal circumstances by expert endoscopists. Lesions that require resection will virtually always be detected on WLE by the expert team performing the work-up endoscopy. Advanced imaging techniques may detect additional flat lesions, inconspicuous with WLE, but these are clinically of limited significance since they harbor only flat type mucosal neoplasia that will be effectively eradicated by ablation therapy.



No endoscopic imaging technique can reliably assess submucosal or lymphangio invasion. Endoscopic resection of early Barrett's neoplasia is therefore imperative for staging and optimal patient selection. Optical chromoendoscopy, such as NBI, plays an important role in delineation prior to endoscopic resection and follow-up after successful ablation therapy. In conclusion, dye-spray chromoendoscopy, optical chromoendoscopy, autofluorescence imaging (AFI) and confocal laser endomicroscopy (CLE) have little clinical relevance for standard surveillance, work-up and treatment of early Barrett's neoplasia.

Part Two: TriModal Imaging and Molecular Endpoints [TIME studies]

Although AFI was shown to have a high false-positive rate, we hypothesized that AFI-positive, dysplasia-negative areas may in fact harbour early molecular changes that predict the presence of, or even progression to early neoplasia. In **chapter 7** we performed a retrospective analysis of AFI guided biopsies and correlated AFI features to a predefined panel of biomarkers; the retrospective TIME study. We selected 58 samples from a previous prospective study on AFI: 15 true-positive (TP: AFI-positive, HGIN/IMC), 21 false-positive (FP: AFI-positive, no HGIN/IMC), 12 true-negative (TN1; AFI-negative, no HGIN/IMC in sample), 10 true-negative (TN2: AFI-negative, no HGIN/IMC in esophagus). Methylation-specific RT-PCR was performed for *HPPI*, *RUNX3*, *p16*, and immunohistochemistry for *Cyclin A*, *p53*. $p < 0.05$ was considered statistical significant. Bonferroni correction was used for multiple comparisons. *p16*, *Cyclin A*, *p53* correlated with dysplasia ($p < 0.01$, $p = 0.003$, $p < 0.001$ respectively). Increased *p16* methylation was observed between TP vs TN2 ($p = 0.003$) and TN1 vs TN2 ($p = 0.04$) subgroups, suggesting a field defect. Only *p53* correlated with AFI-status ($p = 0.003$). After exclusion of HGIN/IMC-samples, significance was lost. We concluded that none of the investigated biomarkers correlated with AFI status. Correlation with dysplasia status was confirmed for *p16*, *Cyclin A* and *p53*, underlining the importance of these biomarkers as an early event in neoplastic progression.

We subsequently evaluated and validated the correlation between AFI characteristics and a larger panel of biomarkers in a prospective multicenter study with short-term follow up, described in **chapter 8**. A cross-sectional prospective study on 175 Barrett's patients was performed in three tertiary referral centers. All patients underwent high resolution endoscopy followed by AFI. Aneuploidy/tetraploidy; 9p and 17p loss of heterozygosity (LOH); *RUNX3*, *HPPI* and *p16* methylation; *p53* and *Cyclin A* immunohistochemistry (IHC) were assessed on biopsies targeted by AFI. Bootstrap resampling was used to select the best predictive biomarker panel for HGIN/IMC.

Aneuploidy, *p53* IHC and *Cyclin A* had the strongest association with dysplasia in the per-biopsy analysis and, as a panel, had an area under the curve of 0.97 (95%CI 0.95-0.99) for the diagnosis of HGIN/IMC. We found biomarker enrichment in AFI-positive compared to AFI-negative areas and the diagnostic accuracy for patients with HGIN/IMC of the 3-biomarker panel from AFI-positive areas was superior to AFI-negative areas ($p < 0.001$). Furthermore, this 3-biomarker panel had equal sensitivity to the standard protocol with a 4.5-fold reduction in the number of biopsies. In an independent cohort of patients, the panel had sensitivity and specificity for HGD/EC of 100% and 90% respectively.

The prospective TIME study demonstrated that the combination of AFI and a 3-biomarker panel provides an objective assessment of the overall dysplasia status with a significant reduction in the number biopsies needed compared to the standard protocol.

Part Three: Back to the basics; probe based fluorescence spectroscopy

In **chapter 9** an optical biopsy system (OBS) was investigated, using a 405nm induced fluorescence spectroscopy probe integrated in a standard biopsy forceps. This allows real-time spectroscopy and ensures spot-on correlation between the spectral signature and corresponding physical biopsy. In this study we aimed to construct an algorithm to distinguish neoplastic from non-neoplastic Barrett's mucosa and to apply this algorithm in the optical biopsy system. In Barrett's patients undergoing endoscopy, areas suspicious for neoplasia and endoscopically non-suspicious areas were investigated with the OBS, followed by a correlating physical biopsy with the optical biopsy forceps. Spectra were correlated to histology and an algorithm was constructed to discriminate between HGIN/IMC and non-dysplastic Barrett's esophagus (NDBE) using smoothed linear discriminant analysis. The constructed classifier was internally cross-validated and correlated to the endoscopist's assessment of the Barrett's segment.

A total of 47 patients were included (39 males, age 66 yrs): 35 patients were referred with early neoplasia and 12 patients with NDBE. A total of 245 areas were investigated with following histology: 43 HGIN/IMC, 66 low-grade intraepithelial neoplasia, 108 NDBE, 28 gastric or squamous mucosa. Areas with LGIN and gastric/squamous mucosa were excluded. The area under the ROC curve of the constructed classifier was 0.78. Sensitivity and specificity of OBS alone were 81% and 58% respectively. When OBS was combined with the endoscopist's assessment, sensitivity was 91% and specificity 50%. If this protocol would have guided the decision to obtain biopsies, half of the biopsies would have been avoided, yet 4/43 areas containing HGIN/IMC (9%) would have been inadvertently classified as unsuspecting. In this study, the feasibility of OBS with the constructed algorithm as an adjunctive tool to the endoscopist's assessment during endoscopic BE surveillance was demonstrated. These results should be validated in future studies. In addition, other probe based spectroscopy techniques may be integrated in this optical biopsy forceps system.

Given the suboptimal performance of (auto)fluorescence imaging, we hypothesized that the excitation wavelengths may be optimized.

In **chapter 10** we describe a custom build multiwavelength fluorescence spectroscopy system, to assess the autofluorescence characteristics of early neoplasia at various excitation wavelengths and correlation to histology. The endoscopic spectroscopy system contained five (ultra)violet light sources ($\lambda_{exc} = 369 \text{ nm}$ to 416 nm) to generate autofluorescence. In Barrett's patients, autofluorescence spectra were collected at areas suspicious and non-suspicious for neoplasia, followed by a biopsy for histological assessment and spectra correlation. Three intensity ratios (r_1 , r_2 , r_3) were calculated by dividing the area, A , under the spectral curve of selected emission wavelength ranges for each spectrum generated by each excitation wavelength λ_{exc} as follows: $r_{1,\lambda_{exc}} = (A_{560\pm5}/A_{640\pm5})$, $r_{2,\lambda_{exc}} = (A_{495\pm5}/A_{560\pm5})$ and $r_{3,\lambda_{exc}} = (A_{495\pm5}/A_{640\pm5})$. We obtained 58 autofluorescence and corresponding biopsy samples out of 22 patients. Excitation with 395 nm, 405 nm or 410 nm showed a significant ($p \leq 0.0006$) differentiation between specialized intestinal metaplasia and grouped high grade dysplasia/early cancer for intensity ratios r_2 and r_3 . Optimal discrimination between non-dysplastic Barrett's mucosa and early neoplasia was achieved using 395 nm excitation and intensity ratio r_3 ($A_{495\pm5}/A_{640\pm5}$) with a sensitivity of 80.0 % and specificity of 89.5 %.



Following this study, we hypothesized that by applying this specific exogenous dye, the fluorescence contrast between normal and neoplastic tissue may be enhanced. However, the excitation spectrum of PpIX was not known for Barrett's mucosa and neoplasia. In **chapter 11**, the multiwavelength fluorescence spectroscopy system was used to study the optimal excitation wavelength of 5-ALA induced PpIX fluorescence in neoplastic and non-neoplastic esophageal mucosa. A chorioallantoic membrane (CAM) model was used to simulate an in-vivo situation. Esophageal adenocarcinoma cells (OE19) and biopsy specimens from Barrett's patients were transplanted onto the CAM of fertilized chickens's eggs. At discrete timepoints, 5-ALA was administered and the cells/biopsy specimens were excited with six light sources (369 nm, 395 nm, 400 nm, 405 nm, 410 nm, 416 nm) sequentially and at several time points between 0 hour before and 28 hours after 5-ALA administration. From all obtained emission spectra the PpIX fluorescence intensity ratio I_{636}/I_{600} was calculated.

Fluorescence spectroscopy before and after 5-ALA administration was performed on 16 OE19 cell samples and 55 biopsy specimens obtained from 17 patients. The optimal wavelength for PpIX excitation in OE19 cells and in Barrett's tissue was determined to be 410 nm and 416 nm. With this information, future fluorescence spectroscopy modalities can be studied in combination with 5-ALA as a contrast enhancer.

Part Four: Optical Frequency Domain Interferometry

In **chapter 12** optical frequency domain interferometry (OFDI) was studied, incorporated in the balloon based volumetric laser endomicroscopy (VLE) system. We hypothesized that with this high-resolution scattering based technique, the esophageal mucosa can be adequately assessed for the presence of neoplasia in real-time. Therefore we aimed to assess the relevant VLE characteristics of neoplastic and non-neoplastic BE both in- and ex-vivo and develop the optimal approach for one-to-one correlation of VLE images with histology. Patients with and without early neoplasia underwent endoscopic resection (ER) of areas marked in-vivo with electrocoagulation markers (ECM). Additional ex-vivo markers were placed using different methods, followed by ex-vivo VLE scanning of the specimen. Tissue blocks were carefully sectioned guided by the placed markers. All histology and VLE slides were evaluated by 2 researchers and considered a match if a) ≥ 2 markers were visible on both modalities and b) mucosal patterns aside from these markers matched on both histology and VLE. All slides were evaluated by an expert BE pathologist. From 16 ER specimens (overall diagnosis: 7 non-dysplastic BE, 9 dysplastic BE (1 LGD, 4 HGD, 4 EAC)) 120 tissue blocks were sectioned of which 57 contained multiple markers and thus could potentially be matched with VLE. Based on several combinations of these markers, a total of 14 histology-VLE matches were constructed. Markers that achieved the best yield of matches were: in-vivo placed ECMs (8 matches with 12 markers), pins (7 with 11), and ink (4 with 5). Histological evaluation was not hindered by marker use. We concluded that one-to-one correlation of VLE and histology is complex but feasible. The groundwork laid in this study will provide high-quality histology-VLE correlations that will allow further research on VLE structures and VLE features of early neoplasia in BE.

One of the possible applications of VLE would be the evaluation of neosquamous epithelium after radiofrequency ablation (RFA) and the possible presence of buried Barrett's. In *chapter 13* we therefore performed in-vivo VLE to detect subsquamous structures suspicious for buried Barrett's in patients with 100% endoscopic regression of dysplastic Barrett's epithelium after RFA. Areas with suspicious subsquamous VLE structures were marked with electrocoagulation, followed by endoscopic resection and ex-vivo VLE scanning of the resection specimen. Extensive histological sectioning was then performed and all histology slides were evaluated by an expert BE pathologist (blinded for VLE images). In 9 patients, 6 areas with suspicious subsquamous structures were seen on in-vivo VLE and resected. Ex-vivo VLE of these 6 ER specimens reconfirmed the presence of these subsquamous structures in 5 ER specimens. Extensive histological sectioning of these areas showed buried Barrett's in one area. The other subsquamous VLE structures corresponded to dilated (ducts of) (sub)mucosal glands or blood vessels. We thus concluded that VLE may potentially detect buried Barrett's under endoscopically normal appearing neosquamous epithelium. However, most post-RFA subsquamous structures identified by in-vivo VLE did not correspond to buried Barrett's.