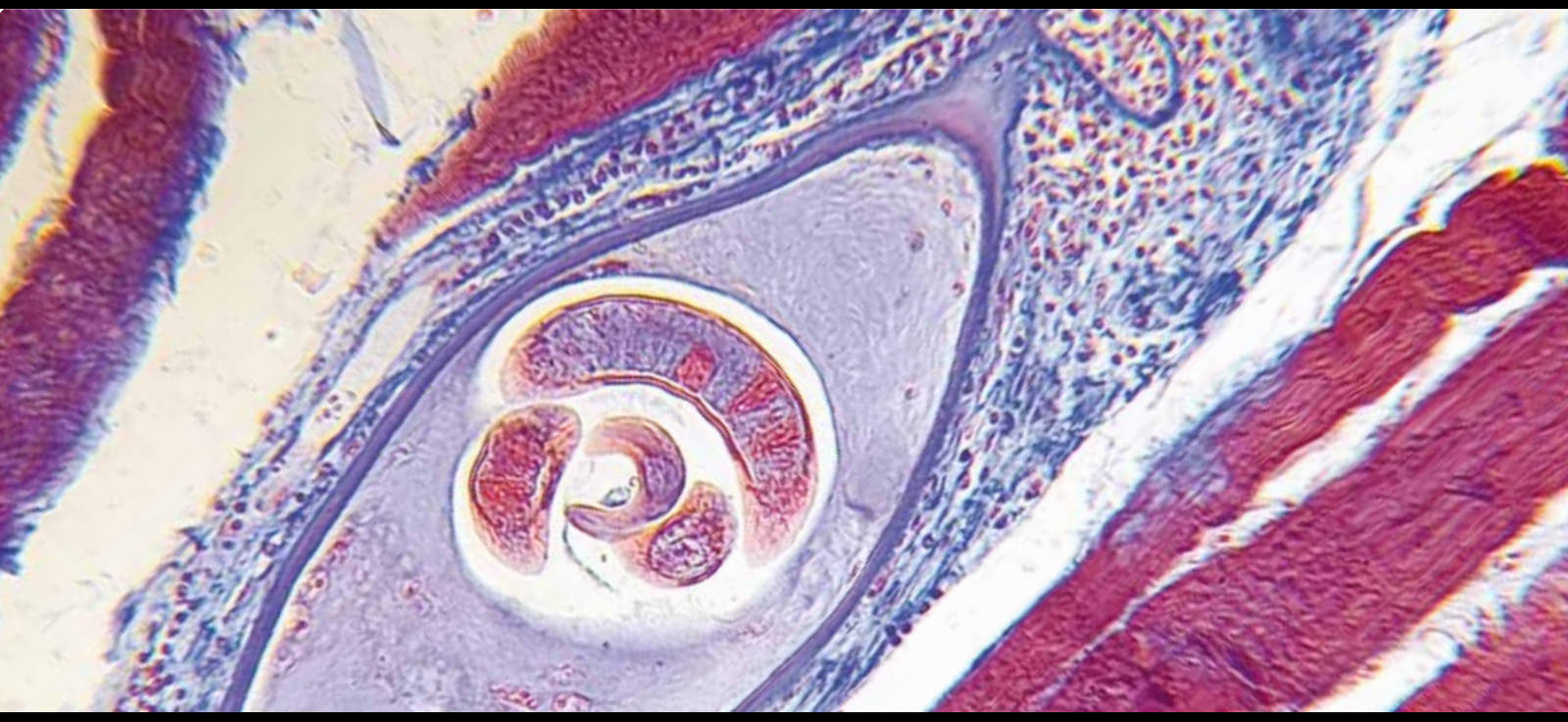
Gastroenterology Research and Practice

Confocal Laser Endomicroscopy

Guest Editors: Giovanni D. De Palma, Michael B. Wallace, and Marc Giovannini



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Editorial Board

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Editorial

Confocal Laser Endomicroscopy

Giovanni D. De Palma,[1] Michael B. Wallace,[2] and Marc Giovannini[3]

[1] *Dipartimento di Chirurgia Generale, Geriatrica ed Endoscopia Diagnostica ed Operativa, Università Federico II di Napoli, Via Pansini, 5, 80131 Napoli, Italy*
[2] *Department of Gastroenterology and Hepatology, Mayo Clinic, Jacksonville, FL, USA*
[3] *Endoscopic Unit, Department of Medical Oncology, Paoli-Calmettes Institute, Marseille, France*

Correspondence should be addressed to Giovanni D. De Palma, giovanni.depalma@unina.it

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Confocal laser endomicroscopy (CLE) is a newly developed endoscopic technique that enables imaging of the mucosal layer during endoscopy at a subcellular level of resolution. The method can therefore be used for the assessment of changes in vascular architecture, connective tissue, and cellular components in the mucosa, enabling endoscopists to collect real-time in vivo histological images or "virtual biopsies" of the gastrointestinal (GI) mucosa during endoscopy.

Confocal microscopy consists of focusing a laser beam (such as an argon-ion laser that generates an excitation wavelength of 488 nm, blue laser light) onto the plane of interest and filtering the returned light by means of a small pinhole which rejects out-of-focus light. The illumination and detection systems are in the same focal plane and are termed "confocal".

After passing the pinhole, the fluorescent light is detected by a photodetection device (a photomultiplier tube or avalanche photodiode), transforming the light signal into an electrical one that is recorded by a computer. All detected signals from the illuminated spot are captured and measured. As the laser scans over the plane of interest, a whole image is obtained pixel-by-pixel and line-by-line, whereas the brightness of a resulting image pixel corresponds to the relative intensity of detected fluorescent light. The gray-scale image created is an optical section representing one focal plane within the examined specimen.

Because confocal images depend on fluorescence, a fluorescent dye (contrast agent) is required to make objects visible. The contrast agents can be applied systemically (fluorescein, tetracycline) or topically (acriflavine, cresyl violet) by using a spraying catheter. Of these, intravenous fluorescein sodium (10%) and topically applied acriflavine (0.2%) have been most commonly used in humans.

CLE can be performed currently with 2 devices: (1) integrated into an endoscope (Pentax, Tokio, Japan, herein termed eCLE) and (2) as a stand-alone probe (herein termed pCLE) capable of passage through the accessory channel of most endoscopes (Cellvizio, Mauna Kea Technologies, Paris, France).

In this special issue on CLE, we have invited a few papers that address the current potential indications for pCLE imaging in clinical gastroenterology and its potential impact in the future, particularly in the screening or surveillance of GI neoplasia.

A paper in this special issue by H. Bertani et al. reviews the role of pCLE in Barrett's esophagus for detection of esophageal dysplasia and carcinoma from a clinical practice perspective. Another paper by Mascolo et al. evaluates the accuracy of pCLE in the detection of aberrant crypt foci (ACF), comparing in double-blind manner the microendoscopic and histopathological features resulting from colonic biopsy. By pCLE, the authors identified specific crypt architecture modifications associated with changes in cellular infiltration and vessels architecture, highlighting a good correspondence between pCLE features and histology.

A paper by V. Ussui and B. Wallace reviews the role of CLE as applied to colorectal polyps detected during colonoscopy. It describes the importance of probe-based confocal endomicroscopy on colorectal polyps with a particular emphasis on distinguishing hyperplastic from neoplastic polyps.

One paper of this special issue, by F. Salvatori et al., reviews the current data on the clinical application of CLE in the study of colonic mucosa in patients with ulcerative colitis.

Finally, a paper, by R. Cannizzaro et al. describes the use of CLE to analyze the angiogenic process in colo4rectal and gastric cancer patients and the possibility of a translational approach combining the confocal imaging with the diagnosis in vivo and the specific molecular profile of the patient with the targeted antiangiogenic treatment.

Giovanni D. De Palma
Michael B. Wallace
Marc Giovannini

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Clinical Study

Probe-Based Confocal Laser Endomicroscopy Evaluation of Colon Preneoplastic Lesions, with Particular Attention to the Aberrant Crypt Foci, and Comparative Assessment with Histological Features Obtained by Conventional Endoscopy

Massimo Mascolo,[1] Stefania Staibano,[1] Gennaro Ilardi,[1] Maria Siano,[1] Maria Luisa Vecchione,[1] Dario Esposito,[2] Gaetano De Rosa,[1,3] and Giovanni Domenico De Palma[4]

[1] *Department of Biomorphological and Functional Sciences, Pathology Section, University of Naples "Federico II", 80131 Naples, Italy*
[2] *Center of Excellence for Technical Innovation in Surgery (ITC), Department of General Surgery, Geriatrics, Oncology, and Advanced Technology, University of Naples "Federico II", 80131 Naples, Italy*
[3] *Oncology Research Center of Basilicata, CROB, 85028 Rionero in Vulture, Italy*
[4] *Center of Excellence for Technical Innovation in Surgery (ITC), Department of General Surgery, Geriatrics, Oncology, and Advanced Technology, University of Naples "Federico II", 80131, Naples, Italy*

Correspondence should be addressed to Stefania Staibano, staibano@unina.it

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The colorectal carcinoma represents one of the most common and aggressive malignancies, still characterized by an unacceptable mortality rate, mainly due to the high metastatic potential and to a late diagnosis. In the last years, the research community focused on the chance of improving the endoscopic screening to detect neoplastic lesions in a very early stage. Several studies proposed aberrant colonic crypt foci as the earliest recognizable step of transformation in colonic multiphase carcinogenesis. We previously demonstrated the clinical applicability and predictive power of probe-based confocal laser endoscopy (pCLE) in superficial colorectal neoplastic lesions and also characterized *in vivo* a case of dysplasia-associated lesional mass (DALM) in ulcerative colitis. Now, we aim to evaluate the accuracy of pCLE in the detection of ACF comparing in double-blind manner the microendoscopic and histopathological features resulting from colonic biopsy. By pCLE, we identified specific crypt architecture modifications associated with changes in cellular infiltration and vessels architecture, highlighting a good correspondence between pCLE features and histology.

1. Introduction

Colorectal cancer represents the third most common human malignancy after prostate and lung cancer in males and the second one after breast carcinoma in females, with more than 1.200.000 new cases [1, 2], constituting a major cause of cancer death worldwide, particularly in Europe where it is responsible for more than 200.000 deaths per year [3]. Although this unacceptable mortality rate is closely associated with high metastatic ability of colorectal cancer, many of the deaths are caused by a late diagnosis. In fact, the prognosis of each malignancy strongly depends on stage at diagnosis and most cancers can be successfully treated if diagnosed at an early stage. For this reason, the research community focused its efforts not only in attempt to better understand the molecular mechanisms underlying the colon carcinogenesis but also on the possibility to improve the endoscopic screening of the colorectal lesions in the very early stage. To date, colon endoscopy remains the best way to make cancer prevention of this district possible. Given

the finding that conventional colonoscopy sometimes is not able to differentiate between neoplastic and nonneoplastic lesions, several studies evaluated the role of advanced new endoscopic imaging techniques, such as chromoendoscopy and confocal laser endomicroscopy (CLE), in the detection of colorectal lesions [4–6]. CLE enables to obtain *in vivo* microscopic images during endoscopy, allowing to make real-time adequate diagnosis and to perform target biopsies improving the diagnostic accuracy. Presently, there are two devices to perform CLE: the endoscope-based confocal laser endomicroscopy (eCLE; Pentax, Tokio, Japan), in which a confocal probe is incorporated in the tip of a routinary endoscope, and the probe-based confocal laser endomicroscopy (pCLE), in which the stand-alone probe can be passed through the biopsy channel of traditional endoscope (Cellvizio, Mauna Kea Technologies, Paris, France) [4, 5, 7]. To date there are not still adequate data to consider an endoscopic technique better than the other one.

It is known that colorectal carcinogenesis is a multistep process progressing through several morphological stages [8]. The earliest phase may be the formation of aberrant crypt foci (ACF). In fact, the ACF prevalence and density are greater in patients with colorectal carcinoma and adenoma, compared to normal controls, and, therefore, these lesions could be used as biomarker of colorectal cancer [9, 10]. Moreover, considering that ACF are preventable preneoplastic lesions and that their growth is modified by specific modulators, their early detection is very important [11]. However, there is still a wide variation of endoscopic criteria useful to identify and define ACF. The main considered feature is the mucosa color [12–15], usually darker compared to the adjacent normal colonic mucosa, but also the crypt architecture, the crypt lumen size [13, 14, 16], the raised appearance [13, 15, 16], and the thickness of epithelial lining [14] are considered. A height of less than 2 mm has been proposed to differentiate them from colonic polyps in some recent studies [17].

In a previous study, conducted as conclusion of MIUR/PRIN project (2007) on this specific topic, we demonstrated that pCLE constitutes a reliable tool for the identification of colorectal superficial carcinoma [4]. In addition, we first discussed the pCLE findings regarding a case of dysplasia-associated lesional mass (DALM) in chronic ulcerative colitis (CUC) [5]. On the basis of these previous reports, we correlate for the first time endoscopic and histological features of ACF in the attempt to validate the promising role of pCLE as useful and predictive tool of evaluation of colorectal preneoplastic lesions.

2. Materials and Methods

2.1. Patients. A small group constituted of 9 patients with evidence of ACF at routine colonoscopy were enrolled for this study. Endoscopic features considered for the identification of ACF, according to the literature data, were darker colonic mucosa after dying, the two-threefold crypt lumen size, raised appearance, and/or thickened epithelium [12–14, 16, 18]. Exclusion criteria were patients aged less than 18 years, known allergic diseases, and impaired renal function. In addition, 5 patients with colonic adenomas and 5 patients with adenocarcinoma at endoscopy were selected. Three hyperplastic polyps were added as control.

2.2. Equipment. Lesions were identified using white-light endoscopy and NBI followed by pCLE imaging recorded by a Coloflex UHD-type probe, using the Cellvizio Endomicroscopy System (Mauna Kea Technologies, Paris, France) [5]. Coloflex UHD-type probe is a 2.5 mm catheter probe inserted through the endoscope-working channel to obtain dynamic images of the mucosa. pCLE imaging data were registered at a scan rate of 12 frames per second with a scanning field of 30,000 pixels. The field of view is of 240 $\times$ 200 μm, with a lateral resolution of 1 μm. From single video frames is reconstructed 1 larger static image (4 $\times$ 2 mm) by a special computer software (mosaicing), which uses a hierarchical framework algorithm able to recover a globally consistent alignment of the input frames, to compensate for motion-induced distortions and to capture nonrigid deformations. The resulting image combines all moving images, cancels motion artifacts, and reconstitutes panoramas of the tissues.

2.3. Procedure. All examinations were performed by a single experienced endoscopist (GDDP). During twenty-four hours before the procedure, 4 L of isotonic polyethylene glycol solution was administered as a bowel cleansing. A conscious sedation with midazolam (5–10 mg i.v.) was administered when requested by the patient. After the identification of each lesion on white-light endoscopy or NBI, a 10–20 mg intravenous bolus of Buscopan (hyoscine-N-butyl-bromide) was given to limit peristaltic artifacts, followed by the administration of 10 mL of 10% sodium fluorescein for CLE image acquisition. Confocal images of circumscribed lesions and four segmental "normal" colorectal quadrants were acquired, the latter used to define normality. Specimens obtained (resected lesions and/or target biopsy) were formalin fixed and paraffin embedded and, then, stained with hematoxylin-eosin. The histologic evaluation was performed by two experienced pathologists (MM and SS) in a blinded fashion and graded in accordance with the Vienna modified classification of gastrointestinal epithelial neoplasia [19]. Histologically, ACF were defined as enlarged crypts (at least 1.5 times larger than normal), covered by thickened epithelium with lack of stratification, but characterized by regular nuclei with only mild or focal crowding, often elevated from adjacent normal mucosa, according to the proposed criteria [20–22].

2.4. Main Outcome Measurements. According to the Paris Workshop guidelines [23], all identified lesions were classified as follows: protruding lesions (Ip: pedunculated polyp; Ips: subpedunculated polyp; Is: sessile polyp); flat elevated lesions (0-IIa: flat elevation of mucosa; 0-IIa/c: flat elevation with central depression); flat lesions (0-IIb: flat mucosal

change; 0-IIc: mucosal depression; 0-IIc/IIa: mucosal depression with raised edge). The diagnostic endoscopic criteria used for diagnosing ACF were crypts larger in diameter than the surrounding normal crypts, from which they are distinguished by deeper color when stained with methylene blue, thicker epithelium and raised appearance [9, 12–14, 16, 24–27]. The endoscopy operator (GDDP) made a preliminary diagnosis based upon the *in vivo* images (video sequences) and the mosaic images, according to the Miami confocal endomicroscopy criteria for the prediction of intraepithelial colorectal neoplasia [28]. pCLE diagnosis was then compared with the histopathological diagnosis. Every image was judged as good, average, or poor by the principal investigator, basing on presence/absence of moving artifacts and on a well/poor recognizable crypt and vascular architecture. To assess interobserver agreement, 50 confocal video images and mosaicing images of good or average quality (25 images of neoplastic lesions and 25 images of nonneoplastic lesions or normal colorectal epithelium) were randomly selected and evaluated in a blinded fashion by one endoscopist (DE) with minimal experience with pCLE. Their prediction of malignant or benign features on pCLE was compared with the histopathologic diagnosis.

3. Results

For this study, 9 patients (4 males, mean age 65 years, range 56–83) with endoscopic evidence of ACF, 5 patients with colonic adenoma (5 males, mean age 60,6, range 49–72), 5 patients with colonic carcinoma (3 males, mean age 63,2, range 56–73) and 3 patients with hyperplastic polyps (2 males, mean age 57,7, range 41–76) were considered. A total of 30 lesions were identified. A single lesion was found in 14 (63,6%) cases, and 8 (36,4%) patients had two lesions. The lesions were located in the rectum in 5 cases, in the sigmoid colon in 4 cases, in the descending colon in 5 cases, and in the right colon in 8 cases.

3.1. Correlation of Histopathology and pCLE Images. On pCLE examination, normal mucosa was defined by a hexagonal, honeycomb appearance with a round crypt structure, surrounded by regular vessels, covered by a homogeneous epithelium with "black-hole" goblet cells in the subcellular matrix; hyperplastic mucosa was characterized by crypts with slit or stellate openings covered by uniform epithelium, with a regular vessel architecture, with some increase in pericryptic capillary density; neoplastic tissue was represented by "dark" cells, with mucin and goblet cell/crypt density depletion; the architectural pattern was irregular, as well as the epithelial thickness, with villiform structures or crypt fusion and distortion, and "dark" epithelial border. The blood vessels were dilated and irregularly branching.

A suspected ACF, identified with traditional endoscopy, can show characteristics of dysplastic adenoma or hyperplastic polyp on pCLE, as previously described.

All pCLE images diagnosed as "normal" mucosa showed normal architecture at histopathologic evaluation. Among cases recorded as ACF at pCLE, histologic evaluation confirmed the presence of aberrant crypts in 7 biopsy specimens and in two of these cases a diagnosis of microadenoma with low-grade displasia was made (Figure 1). Four out of 5 lesions diagnosed as adenoma and 5/5 diagnosed as adenocarcinoma at pCLE showed correspondence at histology. One lesion diagnosed as adenoma on CLE at histologic evaluation was diagnosed as hyperplastic polyp. Hyperplastic polyps used as control and so selected on confocal imaging were confirmed as benign on histology, but in 2 of these features of hyperplasia were showed. No patients showed endoscopic complications or adverse reactions to sodium fluorescein; only a slight yellowish discoloration of the skin was recorded, which usually disappeared within 30–60 min.

4. Discussion

Although to date there is not still a close correspondence between the conventional endoscopic images and histological assessment, this association of tools remains the best way to diagnose accurately and then treat as early as possible many diseases of different district, especially of the colorectal tract, including chronic inflammatory, preneoplastic, and neoplastic diseases. Basing upon the finding that conventional colonoscopy is not always able to differentiate between neoplastic and nonneoplastic lesions, in recent years, several studies highlighted the potential use of confocal laser endomicroscopy (CLE), a new emerging technique, in the screening patients for early colorectal cancer detection and prevention [4–6, 29, 30]. This technique allows to obtain *in vivo* microscopic images during endoscopy, enabling to make real-time diagnosis, and to perform targeted biopsies improving the diagnostic accuracy. These newly developed technologyies have been evaluated for several diseases of different districts, such as lung and bladder [31, 32], and in particular it was suggested that pCLE can be employed in the detection of several gastrointestinal tract diseases. In fact, Wang et al. (2011) in his recent work concludes that pCLE can assess the severity of *Helicobacter pylori* gastritis [33], Meining et al. support that pCLE can be used in the management of indeterminate pancreaticobiliary structures [34], and Gaddam developed six diagnostic criteria to identify dysplasia in Barrett's esophagus [35]. However, there are conflicting advises about the CLE promising utility: in fact, Bisschops in an editorial entitled "*Confocal laser endomicroscopy: finally ready to change clinical practice?*" agreed that the CLE is an innovative imaging tool but not enough to justify its use in a general endoscopy unit [36]. Therefore, advantages and limitations of this novel imaging tool, in particular of the pCLE, need to be acknowledged.

ACF, first reported by Bird [37], are considered the result of first insult in CRC and [8], therefore, represent the putative earliest known morphological precursors to colorectal adenoma, capable of progression to CRC, and a marker of colorectal cancer risk [38–41]. However, there are still conflicting data about ACF meaning: some authors consider ACF as detectable first step of colon carcinogenesis; others do not recognize this role. They are localized colonic

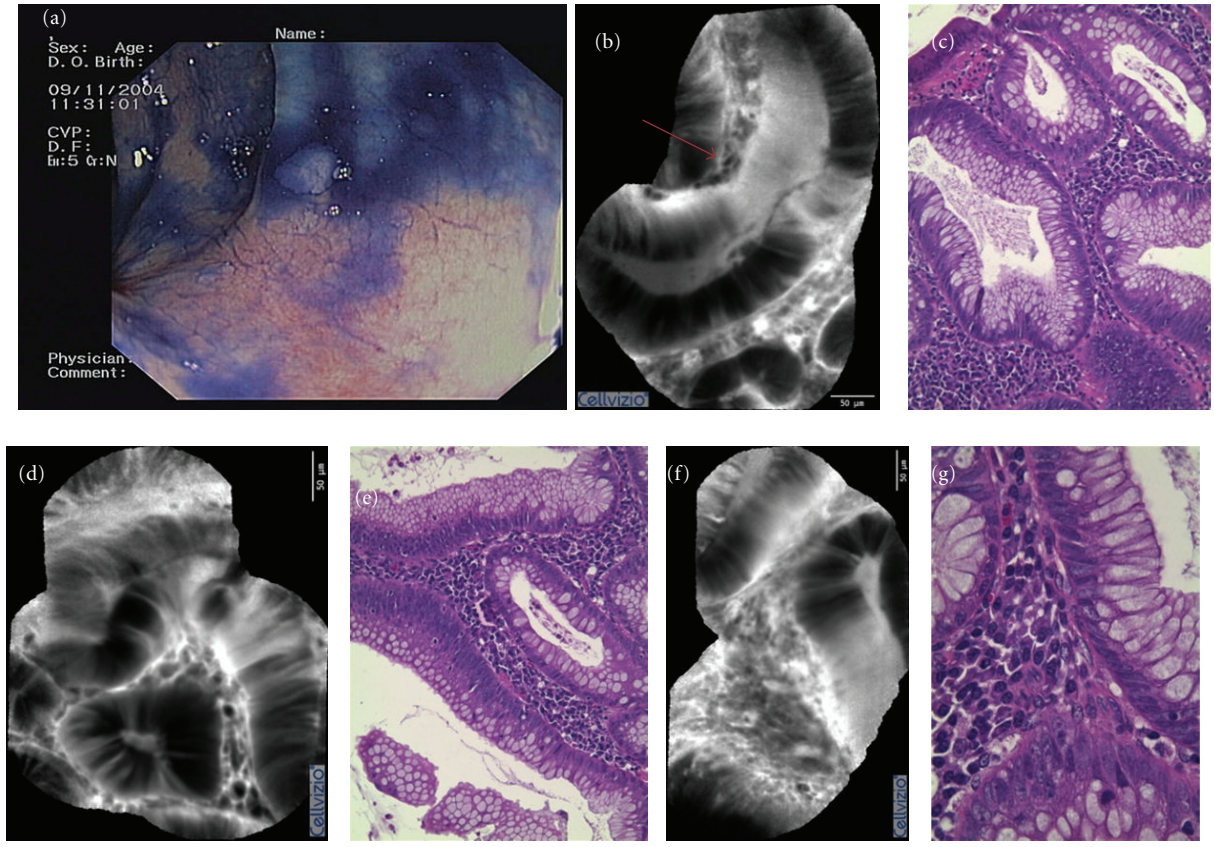

Figure 1: (a) Conventional "white-light" endoscopy of an ACF; (b), (d), and (f) pCLE of the lesion showing an enlarged crypt (red arrow, (b)) and normal globet cell density; (c), (e), and (g) hystologic features of the lesion, showing some enlarged crypts, with thickened epithelium with partial lack of stratification, in the presence of mild dysplasia.

mucosal alterations involving crypts and their surface is epithelium. Histologically, ACF are not specific entity, but morphologically and genetically heterogeneous lesions.

ACF can be identified by high-magnification chromoendoscopy (MCE), but there is still variability in the endoscopic criteria used to define these lesions. The morphologic features most commonly used are darker staining [12–14, 18], larger crypt size [13, 14], raised appearance [13, 16, 18], thicker epithelial lining [14], and dilated crypt lumen [16], compared to the surrounding normal mucosa. In the different studies concerning ACF, a great variability in prevalence [12, 13] and correspondence to histology was found [14, 16]. In fact, data obtained in works using MCE [42] show a prevalence of ACF ranging from 15% [12] to 100% [13] in patients with a normal colon on colonoscopy and from 0% [12] to 61% [16] in patients with sporadic colorectal carcinoma, while the rate of agreement between the endoscopic identification of ACF and histological confirmation ranges from 53% [16] to 92% [14]. This may reflect the actual difficulty in identifying accurately small lesions needed to be biopsied and, more importantly, the necessity to define the endoscopic criteria of ACF [43].

Therefore, in this study we evaluated the correspondence between endoscopic identification by pCLE and histological diagnosis of a small series of ACF, to validate the promising role of pCLE as useful tool in the evaluation of colorectal preneoplastic lesions, in particular ACF. In our work, 7 out of 9 cases (78%) diagnosed as ACF on conventional endoscopy and confirmed on pCLE showed corresponding histological features.

Basing upon our results, we consider a real advantage of the potential of obtaining microscopic images in real time using pCLE because it allows an accurate endoscopic diagnosis and a contemporaneous possibility of treatment, with corresponding time savings and reduced costs of the procedure. Moreover, the "*in vivo diagnosis*" could significantly reduce the number of biopsies to be performed, restricting to those lesions with a real malignant potential, for example, in the management of several chronic diseases, and so limiting the adverse reactions that could occur during multiple randomized biopsy [30]. pCLe major disadvantages are operator dependency regarding the difficulty in maintaining the stability of the probe and in the interpretation of morphologic features and the limited depth of penetration of the tool [6]. This study was designed on the basis of results obtained in two previous works, in which we reported our experience in the identification of superficial colonic neoplasia and in DALM associated with CUC [4, 5]. In fact, we first demonstrated that pCLE has a predictive value of *in vivo* identification of colorectal preneoplastic and neoplastic

lesions [4]. In the second one, instead, we showed the switch from the inflamed to neoplastic mucosa in a patient with chronic ulcerative colitis (CUC) [5]. We confirmed previously reported data, according to Kuiper's work [44], in which they also proposed a new pCLE colon classification, highlighting the high level of accuracy of pCLE in identifying colonic intraepithelial neoplasia. Furthermore, we demonstrated the clinical applicability and predictive power of pCLE also in a group of aberrant colonic crypt foci (ACF) collected during laser confocal endomicroscopy, through the concordance between endoscopic and histological features (rate of architectural alterations in the absence or presence of epithelial dysplasia).

5. Conclusion

To the best of our knowledge, this work constitutes the first attempt to correlate the identification of ACF by pCLE with the actual putative advantages, both in terms of final diagnosis and the concern of the compliance of patients for the endoscopy procedure. Although this study considered only a limited number of patients, the results obtained allow us to suppose that this endoscopic image technique is extremely useful in the identification of these putative very early colonic preneoplastic lesions. This leads to several considerations: the introduction of this imaging technique in an endoscopy unit allows to save time, decreasing both the risk for patients during colonoscopy and the procedure's costs. These data will be validated in future studies on a significantly larger study population. However, these preliminary findings support the idea that pCLE may significantly improve our chances to morphologically specifically detect the colon areas corresponding to ACF, thus increasing the diagnostic accuracy.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgment

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Review Article

Advances in Endoscopic Visualization of Barrett's Esophagus: The Role of Confocal Laser Endomicroscopy

Helga Bertani, Flavia Pigò, Emanuele Dabizzi, Marzio Frazzoni, Vincenzo Giorgio Mirante, Mauro Manno, Raffaele Manta, and Rita Conigliaro

Endoscopy Unit, Nuovo Ospedale Civile S. Agostino Estense, Via Giardini 1355, Baggiovara, 41126 Modena, Italy

Correspondence should be addressed to Helga Bertani, helga88@libero.it

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Many endoscopic imaging modalities have been developed and introduced into clinical practice to enhance the diagnostic capabilities of upper endoscopy. In the past, detection of dysplasia and carcinoma of esophagus had been dependent on biopsies taken during standard white-light endoscopy (WLE). Recently high-resolution (HR) endoscopy enables us to visualize esophageal mucosa but resolution for glandular structures and cells is still low. Probe-based confocal laser endomicroscopy (pCLE) is a new promising diagnostic technique by which details of glandular and vascular structures of mucosal layer can be observed. However, the clinical utility of this new diagnostic tool has not yet been fully explored in a clinical setting. In this paper we will highlight this new technique for detection of esophageal dysplasia and carcinoma from a clinical practice perspective.

1. Introduction

Esophageal adenocarcinoma has the fastest growing incidence rate (300% increase over the past 4 decades) [1–3], and the risk of patients with Barrett's Esophagus (BE) of developing adenocarcinoma is 30–120 times greater. BE is a change in the distal esophageal epithelium of any length that can be recognized as columnar-type mucosa at endoscopy and is confirmed to have intestinal metaplasia by biopsy of the tubular esophagus [4]. The currently accepted paradigm correlates the risk of progression to the grade of dysplasia as there is evidence that progression occurs in an orderly fashion from no dysplasia to low-grade dysplasia (LGD) to high-grade dysplasia (HGD) followed by early esophageal adenocarcinoma [5]. In addition esophageal adenocarcinoma has a poor survival rate (<5% at five years) due to a late diagnosis, to an early vascular and lymphatic infiltration and to low vascularization of neoplastic tissue, which leads to a low response to chemotherapy of the tumor [4].

Since BE is considered the most important risk factor for the development of esophageal adenocarcinoma, assuming that the detection of mucosal dysplasia is critically important in patients with Barrett's oesophagus, because early diagnosis can prevent the progression to invasive carcinoma, international societies of gastrointestinal diseases suggest keeping patients in endoscopy surveillance program [5–7].

However, surveillance endoscopy has several limitations because dysplastic changes occurring in BE are not easily identifiable by standard endoscopy.

In the last decades, many technologic advances have been done in the field of endoscopic imaging, through HR endoscopy to magnification endoscopy to virtual chromoendoscopy, in order to achieve a better visualisation of mucosal layer and to distinguish neoplastic versus nonneoplastic tissue. But even if good, new techniques are not strong enough to replace biopsies. Consequently, the current standard of endoscopic practice is to take multiple biopsies because there are no features on standard or HR endoscopy that distinguish Barrett's glandular metaplasia, dysplasia, or early-stage neoplasia. However the accuracy of standard white light endoscopy (WLE) and random biopsies is low and may fail to detect neoplastic lesions [7]. Moreover biopsies obtained using this technique are prone to sampling error, and interobserver agreement is low even between advanced operators and even among expert pathologists

[8, 9]. This results often in: (1) delay in reaching the final diagnosis and the decision of the correct and best treatment, (2) increased costs in pathology procedures, and (3) repeated procedures. In addition, sensitivity and specificity of histology are variable for difficulty to reach specimen adequacy. Moreover the presence of inflammation or ulcers could alter the mucosal architecture and give some false negative/positive results to pathology examination [10–14]. A multiple biopsies protocol could also interfere with next therapeutic steps; endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD) could be more difficult without adequate "lifting sign" due to scar tissue after repeated biopsies.

Nevertheless another important limitation of histology is that it is a postmortem analysis and it is not able to give us information about in vivo processes (blood flow).

Confocal laser endomicroscopy (CLE), a recent advance of endoluminal imaging, allows an in vivo visualization of mucosal layer with a detailed visualization of tissue and subcellular structures with magnification up to 1000 times. Since 2004 many papers, about the potential role of this new technique, have been published, and many studies have been introduced to validate this technique.

CLE has the potential to anticipate the final diagnosis (neoplastic versus nonneoplastic) and potentially to guide next therapeutic steps in clinical practice without the delay of a pathology response. Moreover it offers the possibility to study mucosal layer to a micron resolution giving us an "optical biopsy". However, other technologies, such as narrow band imaging (NBI), autofluorescence imaging (AFI), and chromoendoscopy, are needed as "red-flag" techniques to initially detect and localize suspicious areas.

One of the potentially future applications of pCLE is about a role in in vivo study of physiologic and pathologic processes, like inflammation or angiogenesis in healthy or neoplastic tissue [15].

Currently two devices are available and approved by European Medicines Agency (EMA) and Food and Drug Administration (FDA) to perform CLE: one system is inserted in tip of the scope (eCLE, Pentax Corporation, Tokyo, Japan) and one, a probe-based system, is a separate device from the endoscope but able to be introduced in the working channel of any standard endoscope (pCLE, Cellvizio, Mauna KeaTech, Paris, France).

eCLE. In this system, the miniaturized confocal scanner has been integrated into the distal tip of a new endoscope. A blue laser light source delivers an excitation wavelength of 488 nm and light emissions detected at >505 nm. Successive points within the tissue are scanned in a raster pattern along X-axis and Y-axis to construct serial en face optical section of $475 \times 475\,\mu$m at user-controlled variable imaging depth. The optical slice thickness is $7\,\mu$m with a lateral resolution of $0.7\,\mu$m. Images on the screen approximate a 1000-fold magnification of the tissue in vivo [16]. The advantage of this system is that the working channel of the scope is free, and it can be used for target biopsies or for combined enhancement techniques such as chromoendoscopy. The limit of this system is that the calibre of the scope is bigger than a standard 11.8 mm upper scope and is stiff. Moreover the lens of the scope is not combined with HR software and virtual chromoendoscopy or other system (ISCAN).

pCLE. this system can be used through the working channel of any standard endoscope (colonoscope, gastroscope, cholangioscope, bronchoscope, and ureteroscop, etc). The advantage of this probe-based CLE is the versatility of the system and the possibility to combine it with other advanced "red flag" imaging modalities such as virtual chromoendoscopy or magnification. Scanning rates is 12 images/sec. The limits of this system pCLE are the slightly low power resolution compared to eCLE (1 mm versus 0.7 mm) and a small field of view (240–600 mm). So pCLE system is not well suited to surveying large areas of tissue such as long segments of BE and should ideally be combined with a red-flag technique for classification of tissue in a site already detected by enhanced endoscopy. However Mauna Kea has developed a postacquisition specifically-developed software ("mosaicing") to paste images together and to obtain images similar to histology specimen.

2. Classification

An important issue for a new imaging technique is the standardization of terminology and classification of images. The first published classification about confocal imaging was the "Mainz classification" based on eCLE [17]. However, due to several technical differences between pCLE and e-CLE, in 2011 a new classification based on pCLE has been published after a consensus of pCLE experts held in Miami in 2009 [18].

pCLE shows detailed images including squamous epithelium, glandular architecture, crypts, columnar cells, goblet cells, and capillaries with red blood cells. In patients with a normal squamous epithelium pCLE shows flat cells without crypts, or villi, bright vessels within capillary loops. In case of BE diagnosis, pCLE shows the villiform architecture, columnar cells, and the presence of goblet cells (Figure 1). If the BE is complicated with dysplasia pCLE shows villiform structures with dark, irregularly thickened epithelial borders, dilated irregular vessels. In case of adenocarcinoma disorganized/loss of villiform structure and crypts dark columnar cells and dilated irregular vessels are found (Figure 2) [18].

3. Barrett's Esophagus Surveillance

One of the first and major clinical applications of pCLE is BE surveillance or, with a therapeutic approach, the definition of lesion's margin before EMR or ESD.

First published data (noncontrolled trials) showed that pCLE was able to detect intraepithelial neoplasia with a sensitivity of 75% and specificity of 89–91% [19]. In the same paper, ranking study population for disease-risk, in the low-risk group population, pCLE has a NPV nearly 98.8% suggesting the possibility to avoid random biopsies. However, a false-positive rate for suspected dysplasia of 64.3% using endomicroscopy was commented, and the authors

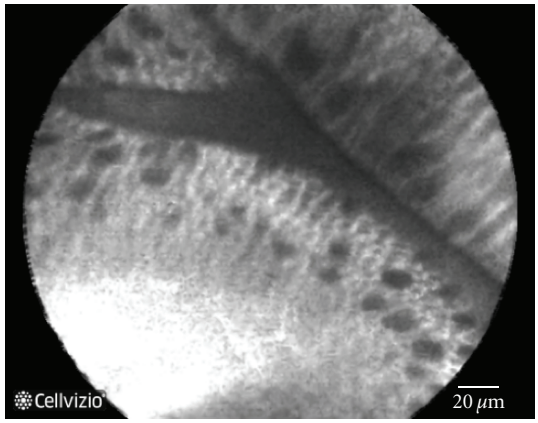

Figure 1: Normal Barrett's mucosa at pCLE: Villiform structure with goblet cell and regular epithelial lining.

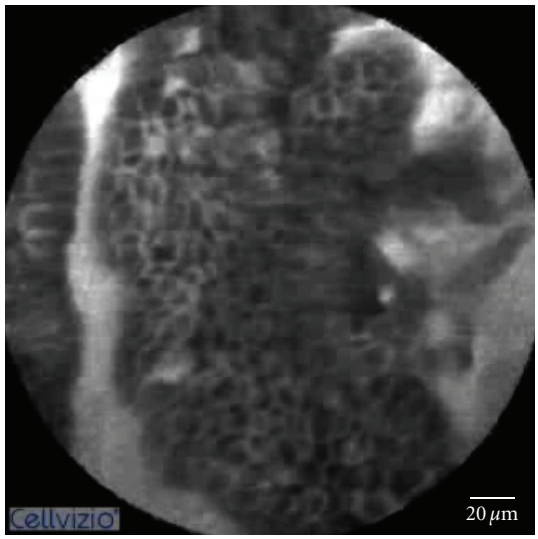

Figure 2: Dysplastic Barrett's mucosa. Villiform structure with dark and irregularly thickened epithelial lining.

replied that the study was not designed to calculate the performance characteristics (sensitivity, specificity, accuracy) [20]. In a pilot study by Pohl et al. the author evaluated the preliminary accuracy of pCLE for high-grade dysplasia (HGD) and adenocarcinoma in BE patients. They evaluated 296 sites in a 38-patient group. The overall accuracy of pCLE was 88% to 93% with a sensitivity of 75% to 80% and specificity of 89% to 94%, a PPV of 44.4%, and a NPV of 98.8% [21]. Kiesslich et al. using the eCLE system achieved an accuracy of 96.8% in BE metaplasia diagnosis and an accuracy of 97.4% in neoplasia diagnosis [17].

Another study by Bajbouj et al. [22] did not confirm these data, and the authors explained the differences with previous results with the low frequency of neoplasia detected in the study and secondly to strict adherence with diagnostic criteria for neoplasia in their data. The prevalence of neoplasia was lower than in the published data using the CLE system or other studies evaluating different imaging modalities, which have described prevalence of HG dysplasia or early cancers ranging between 24% and 59%. The authors also face the problem of overinterpretation in those studies, and the possibility of false positive pCLE findings increases [20].

Dunbar et al. [19] also demonstrated an increased yield of neoplasia compared to 4-quadrant biopsy protocol. Although the sensitivity of pCLE found in this study was lower than found in some previous studies, this study used a real-time prediction of histology by a great number of endoscopists from different Endoscopic Academic centers, which used different criteria in pCLE imaging interpretation, which may have led to variable results.

Recently Sharma et al. published the first international multicenter prospective randomized controlled trial [23]. The authors demonstrated significantly improved sensitivity in detection of HGD/EC using pCLE. The study involved 5 international centers, and 122 patients were enrolled. Of the enrolled patients, 21 were excluded from the analysis. A twofold increased sensitivity in detections of HGD/EC for HD-WLE compared to HD-WLE or pCLE (34.2% to 68.3%, resp.) was shown. This translated into 41 additional locations with HGD/EC being identified when pCLE was used in conjunction with HD-WLE compared with HD-WLE alone [23].

4. Barrett's Esophagus Treatment

Another recent application of confocal endomicroscopy is a role in therapeutic endoscopic procedures. The management of BE with neoplasia (HGD/"early cancer") ranges from surgery to endoscopic treatment and is evolving to include multiple endoscopic modalities in order to increase the rates of esophagus-sparing therapies. Recently, the trend is not only to treat the dysplastic BE but also to eradicate the remaining at-risk Barrett's epithelium to prevent metachronous and synchronous lesions. Endoscopic therapies include both tissue-acquiring (EMR, ESD) and non-tissue-acquiring therapies (RFA and photodynamic therapy). The results of the above-mentioned therapies are very different in the published series; EMR demonstrated a long-term experiences and good results in terms of radicality at the expense of high complications rates such as perforations and strictures [24, 25]. RFA and cryotherapy offer promising results, though the latter still lacks significant follow-up time [26]. But in this great number of emerging therapies, the appropriate selection of an endoscopic modality for the treatment of a lesion in clinical practice is based upon endoscopist's experience and new technologies' availability. pCLE can play a role in (a) localization of lesions and prediction of pathology, (b) in targeting biopsies and resections in surveillance and treatment, and (c) in the choice of therapy to use. Konda et al. recently published a case series [27] showing a possible role of pCLE in therapeutic endoscopy. This case series illustrates a range of cases in which CLE was used during the procedure and offered the chance of providing real-time information during endoscopic treatment or follow-up. Thus, the endoscopist may

have endomicroscopic information, and in the future pCLE could be used to tailor BE management strategy between surveillance, biopsies, ablation, or resection-based strategies.

5. Interobserver Agreement and Learning Curve

Issues to consider before introducing a new technique in clinical practice include the learning curve for the endoscopist and interobserver agreement.

Wallace et al. [28] evaluated the accuracy and interobserver agreement of 9 international endoscopists in pCLE in patients with BE-associated dysplasia. The overall accuracy of pCLE for the diagnosis of HGD was 90.5%, sensitivity 88%, and specificity 94%. If endoscopist had previous experience in endomicroscopy imaging interpretation, the overall accuracy was of 97%, sensitivity 94%, and specificity of 100%. The overall interobserver agreement was 0.72; 95% (CI 0.57–0.85). A matter of contention is the need of a real-time interpretation that may be different from reviewing sequences after image acquisition.

6. Conclusions

The promising results recently published are potentially changing the role of endoscopist into an "endomicroscopist". However, for any emerging technique, more data are needed to confirm the results in clinical setting, to evaluate the possible increased diagnostic accuracy of HR endoscopy and the possibility to reduce the missing rate of dysplastic BE in surveillance program. Another issue will be the applicability of a new technique outside research program and the costs, if applied, in surveillance program.

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Review Article

Confocal Laser Endomicroscopy in the Study of Colonic Mucosa in IBD Patients: A Review

Francesca Salvatori, Saverio Siciliano, Francesco Maione, Dario Esposito, Stefania Masone, Marcello Persico, and Giovanni D. De Palma

Department of General Surgery Geriatrics Oncology and Advanced Technology, Center of Excellence for Technical Innovation in Surgery (ITC), Section of Diagnostic and Therapeutic Endoscopy, School of Medicineand Surgery, University of Naples Federico II, 80131 Naples, Italy

Correspondence should be addressed to Giovanni D. De Palma, giovanni.depalma@unina.it

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Confocal laser endomicroscopy (CLE) is one of several novel methods that provide real-time, high-resolution imaging at a micronscale via endoscopes. CLE and related technologies are often termed "virtual biopsy" as they simulate the images seen in traditional histology. Recently, the use of CLE was reported in the study of colonic mucosa in patients with inflammatory bowel diseases and in particular in patients affected by ulcerative colitis. CLE has the potential to have an important role in management of IBD patients as it can be used to assess the grading of colitis and in detection of microscopic colitis in endoscopically silent segments. Moreover, CLE can be used in surveillance programs especially in high-risk patients. This report aims to evaluate the current data on the application of confocal endomicroscopy in clinical gastroenterology and particularly in the study of colonic mucosa in UC patients.

1. Introduction

Endoscopy has a recognized important role in diagnosis and management in inflammatory bowel disease (IBD).

It can distinguish between Crohn's disease (CD) and ulcerative colitis (UC), assess activity, extension of the disease, and response to therapy, and it permits surveillance, especially in long-standing UC and extensive CD colitis patients, for cancer and dysplasia.

Mucosal biopsy is a critical component of endoscopic examination for patients with suspected IBD to differentiate IBD from other causes of colitis such as bacterial infection, ischemia, and NSAID use; biopsy specimens can help differentiate CD from UC. Mucosal biopsy also helps to establish the extent of colon that is inflamed, which aids in determining prognosis, directing appropriate medical and surgical therapy and stratifying risk for dysplasia.

Moreover, histological findings have an important role in predicting relapse, because patients with acute inflammatory infiltrates seen on histological assessment are more likely to experience relapse than are those without infiltrates. Furthermore, some studies suggest that severity of inflammation is a risk factor for colorectal neoplasia in UC [1].

Colonoscopy underestimates the extent of disease compared with histology, and at present the extent of colitis (pancolitis, left-sided colitis, or proctitis) should be based on histologic examination rather than on endoscopy. Furthermore the assessment of inflammation activity by conventional colonoscopy is inaccurate in the prediction of acute inflammation in some cases, especially for those seeming to be in remission as evaluated by conventional colonoscopy.

Individuals with long-standing UC and extensive CD colitis are at increased risk for development of dysplasia and colorectal cancer (CRC) and should undergo colonoscopic surveillance. Surveillance of patients with ulcerative colitis consists of taking targeted and random biopsies. Biopsy specimens of the colon in patients with documented pancolitis should be obtained in all 4 quadrants every 10 cm from the cecum to the rectum, to obtain a minimum of 32

biopsy samples. Biopsy specimens should be obtained from strictures, mass lesions, and macroscopic abnormalities. The presence of high-grade dysplasia or multifocal low-grade dysplasia in flat mucosa and dysplasia-associated lesional mass (DALM) is an indication for colectomy.

Taking many biopsies is time consuming, carries a low but non negligible risk of secondary hemorrhage, and has only moderate sensitivity for neoplasia detection especially when random biopsies are taken.

In recent years many efforts have been done to improve the diagnostic power of endoscopy, and technology has provided the endoscopist new advanced tools such as chromoendoscopy, high-resolution and magnification endoscopy, narrow-band imaging and autofluorescence.

These new technologies offer enhanced endoscopic images that can predict the histopathological diagnosis of the examined mucosa and target the biopsy to suspected and representative spot of the mucosa. Reported results have shown that these procedures had a better relation with histology than did conventional colonoscopy. However, some impractical aspects of dye-based chromoendoscopy, such as longer procedure times and different dye stainings and washing techniques, contributed to its limited application. Although these factors do not affect the "virtual" chromoendoscopy methods, such as narrow-band imaging (NBI) or Fujinon intelligent color enhancement (FICE), an extensive review by the ASGE on these methods shows modest and variable accuracy [2].

Confocal laser endomicroscopy (CLE) is a newly introduced technique which provides real-time high-magnified images of the gastrointestinal mucosa during endoscopic examination. It offers the chance to the endoscopist to have in vivo visualization of the histology of the mucosal epithelium with its cellular and subcellular structures. CLE during endoscopy has shown high agreement with the real histology of the tissue. The current potential indications for CLE imaging are broad and include almost all the cases in which endoscopic biopsy is needed [1, 3–5].

To date various studies have addressed the potential of CLE in UC patients evidencing that this technique can have an important role in assessing the extension and the activity of disease and in targeting biopsies, reducing the number of useless biopsies and improving the early detection of dysplasia.

In this paper, we will focus on the role of CLE as applied to UC patients with a particular emphasis on the potential of CLE in in surveillance programs.

2. Confocal Systems/Methods

CLE can be performed currently with 2 devices: one integrated into an endoscope (Pentax, Tokyo, Japan, herein termed eCLE) and one as a stand-alone probe (herein termed pCLE) capable of passage through the accessory channel of most endoscopes (Cellvizio, Mauna Kea Technologies, Paris, France).

The Pentax confocal endoscopes (Pentax EG-3870CIK upper endoscope and EC3870CILK colonoscope) generate simultaneously endoscopic and confocal images, so the endoscope working channel is available to use and can capture images at different depth levels from 0 to 250 μm.

The Cellvizio endomicroscopy system probe can be used with any endoscope through the working channel and has different probes which enable different scanning depth levels (40–70 μm, 70–130 μm-55–65 μm). Single video frames are reconstructed by a special computer algorithm (mosaicing) in an image with an enlarged field of view. The video mosaicing technique as applied is based on a hierarchical framework algorithm that is able to recover a globally consistent alignment of the input frames, to compensate for motion-induced distortions. The resulting video mosaics combine all moving images, cancel motion artifacts, and reconstitute panoramas of the tissues.

There are no data, at present, comparing pCLE with eCLE to demonstrate the superiority of any single system. pCLE has several advantages and disadvantages compared with eCLE. Advantages include the greater versatility of pCLE probes, which can be used in conjunction with virtually any endoscope (high-resolution endoscopes, NBI, cholangioscope, etc.), ad hoc usage (such as when a lesion is detected with a normal endoscope), and acquisition at video frame rate of 12 frames/s, allowing in vivo imaging of capillary flow. Disadvantages include a slightly lower resolution (approximately 1 μm compared with 0.7 μm for eCLE) and smaller field of view (240–600 μm).

Unequivocally, this technology is best used in conjunction with other "red-flag" techniques because of its minute scanning area and thus is only appropriate for classification of tissue at a site already detected by standard or optically enhanced endoscopy. Ideally, the "red-flag" techniques such as chromoendoscopy, narrow-band imaging, or autofluorescence imaging should be used to screen the mucosa for "areas of interest," which can then be interrogated by CEM for a "histological" diagnosis. The best combination in UC surveillance is between chromoendoscopy and CLE as chromoendoscopy is the gold standard to detect regions of suspicion that can be examined by CLE to confirm intraepithelial neoplasia and guide immediate therapy.

A fluorescent contrast agent is needed to achieve high-contrast images using CLE. Potentially suitable agents in humans are fluorescein, acriflavine, tetracycline, or cresyl violet. The most commonly used in studies have been fluorescein and acriflavine. Topical acriflavine is highly specific for labeling acidic constituents staining cellular nuclei of superficial layers of the mucosa and may allow better differentiation between intraepithelial neoplasia and cancer of the GI tract. However, because of the risk of mutagenesis related to this agent, its use in humans has been reduced.

Sodium fluorescein is the agent of choice as it is non-mutagenic and relatively inexpensive and it has been safely used for decades in ophthalmology.

It is highly safe with most common side effects being short-term yellowish skin discoloration and bright-yellow-colored urine. Transient and minor nausea and vomiting were reported during angiography. Serious side effects, such as anaphylaxis or cardiac or respiratory effects, are extremely rare, and to date, have not been recorded in CLE.

Table 1: Classification of crypt architecture by e-CLE assessment in ulcerative colitis [6].

CLE crypt architecture	Description
(A) normal	Regular arrangement and size of crypts
(B) chronic inflammation	Irregular arrangement of crypts, enlarged spaces between crypts
(C) acute inflammation	Dilation of crypt openings, more irregular arrangement of crypts, and enlarged spaces between crypts as compared to type B
(D) acute inflammation	Crypt destruction and/or crypt abscess

Intravenous injection of 1.0–5.0 mL of a 10% solution enables visualization of individual cells with strong contrast of the capillary network. Cell nuclei and mucin are not stained by fluorescein and therefore appear dark.

Fluorescein, after binding serum albumin and staining the vascular space, diffuses in the extravascular space and stains the epithelium and the stromal tissue allowing visualization of enterocytes, cellular infiltrate, surface epithelial cells, blood vessels, and red blood cells.

3. Confocal Images Evaluation/Classification

Kiesslich et al. in 2004 were the first who defined criteria for classification of e-CLE patterns of normal, regenerative, and neoplastic tissue based on evaluation of crypts and vascular architecture, named the Mainz confocal endomicroscopy criteria [7]. The Miami classification system was developed for p-CLE images, on the base of a consensus of p-CLE users reached during a meeting held in Miami, Florida, in February 2009 [8]. Due to the significant technical differences compared with e-CLE (smaller field of view, fixed depth), p-CLE images are not comparable to e-CLE images.

At present there is not a worldwide accepted classification of CLE images in UC, and this is certainly a limit of this technique. This reflects the fact that this is a recently introduced technology, and few centers have published studies on this technique up to now. The most used classifications are showed in Tables 1, 2, and 3 and are based on crypt architecture assessment and microvascular assessment [6, 9, 10].

The most frequent alterations in crypt architecture are represented by dilation of crypt openings, more irregular arrangement of crypts, enlarged spaces between crypt, crypt destruction and/or crypt fusion, and crypt abscess with fluorescein leaks into the crypt lumen (therefore making the lumen brighter than the surrounding epithelium) (Figures 1 and 2). Microvascular alterations are mainly represented by dilated, prominent branching vessels.

Dysplasia is characterized by "dark" cells, with mucin depletion and goblet cell/crypt density attenuation; the architectural pattern is irregular, as well as the epithelial thickness, with villiform structures and "dark" epithelial border. The blood vessels are dilated and irregularly branched, with poor orientation to adjunct tissue and fluorescein extravasation (Figures 3 and 4).

Table 2: Microvascular architecture by e-CLE assessment in ulcerative colitis [7].

Vessel architecture	Description
Normal	Hexagonal, honeycomb appearance that presents a network of capillaries outlining the stroma surrounding the luminal openings of the crypts
Inflammation-regenerative	Preserved hexagonal, honeycomb appearance with a slight increase in the number of capillaries
Dysplastic	Dilated and distorted vessels with increased leakage; irregular architecture, with little or no orientation to the adjoining tissue

Table 3: Assessment of crypt architecture and vessel architecture by p-CLE in ulcerative colitis [8].

Crypt architecture	Crypt fusion and distortion Bright epithelium
Vessel architecture	Dilated, prominent branching vessels

4. Clinical Application and Review of the Literature

CLE has the potential to have an important role in management of IBD patients. It cannot distinguish between CD and UC as it cannot be used to make a diagnosis, but it can assess the grading of colitis and detect microscopic colitis in endoscopically silent segments. Moreover, CLE can be used in surveillance programs especially in high-risk patients.

At present most of the literature is about the use of CLE in UC patients, to monitor disease activity and for surveillance.

Watanabe et al. [9] and Li et al. [6] reported on real-time inflammation activity assessment by CLE. The inflammation activity assessment includes crypt architecture, cellular infiltration, and vessel architecture. These studies evidenced that images taken with the CLE provided information that was equivalent to conventional histology, differentiating between active and nonactive CUC patients during ongoing endoscopy.

CLE may be useful particularly in the surveillance of patients with UC, where suspicious lesions can be evaluated in vivo, reducing the need for random biopsies by combining CLE with chromoendoscopy.

Several randomized studies have shown that targeting biopsies with chromoendoscopy significantly increases dysplasia detection rates in patients with long-standing ulcerative colitis [11, 12]. Chromoendoscopy was demonstrated, in fact, to have a higher sensitivity than conventional white light colonoscopy in the detection of dysplasia in UC patients while it has a low specificity. CLE has a high specificity so it would be ideal to join the two techniques in cancer and dysplasia surveillance.

In a randomized study on 161 patients with long-term UC, pan-chromoendoscopy has been used to detect flat or suspected lesion and targeted CLE of the detected lesions

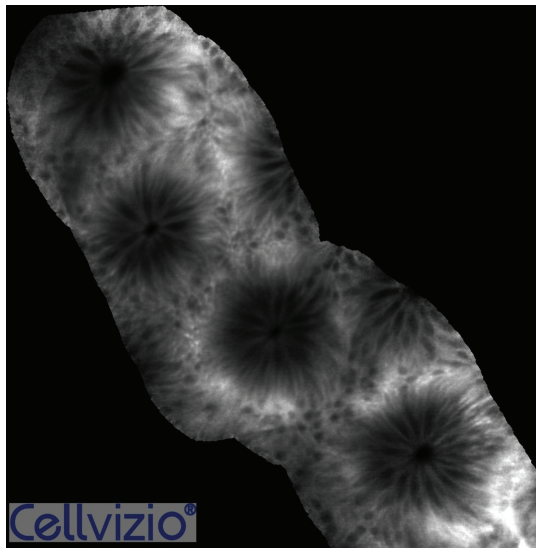
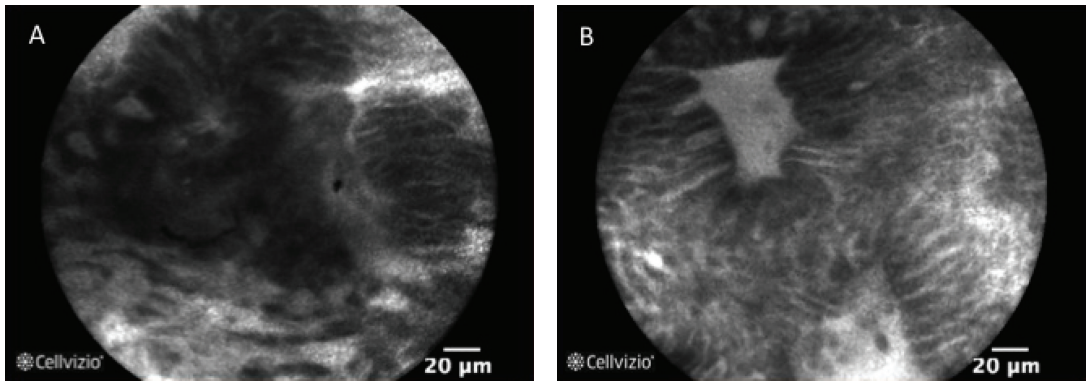


Figure 1: p-CLE fluorescein sodium 10% imaging of the normal colon showing hexagonal, honeycomb appearance with a regular-ordered network of capillaries demarcating the luminal crypt orifice. Surface crypt architecture was classically represented by ordered and regular crypt orifices covered by a homogeneous epithelial layer with visible "black-hole" goblet cells within the subcellular matrix.



(a) (b)

Figure 2: p-CLE fluorescein sodium 10% imaging examples of crypt types of patients in active ulcerative colitis. (a) crypt fusion and distortion; (b) dilation of crypt openings, with fluorescein leaks into the crypt lumen therefore making the lumen brighter than the surrounding epithelium.

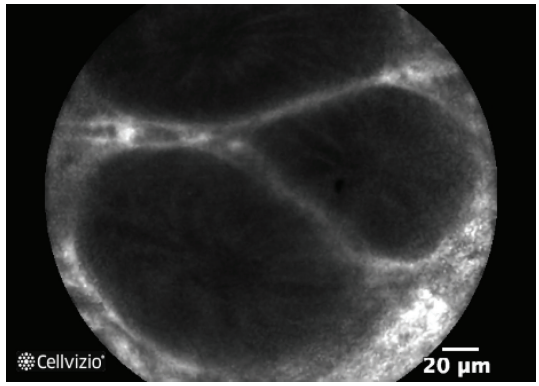
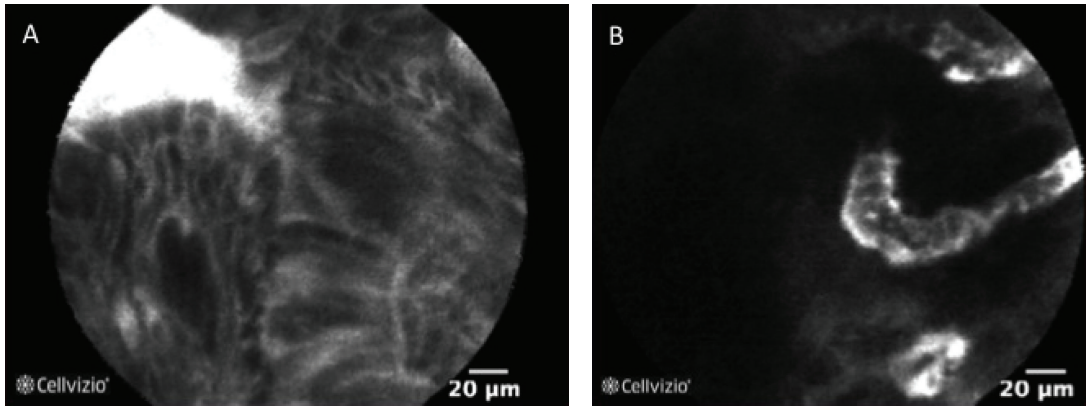


Figure 3: p-CLE fluorescein sodium 10% imaging of vessel architecture in ulcerative colitis: preserved hexagonal, honeycomb appearance with slightly dilated capillaries.



(a) (b)

Figure 4: p-CLE fluorescein sodium 10% imaging of dysplastic epithelium in ulcerative colitis. (a) the architectural pattern of crypt is irregular, with epithelial thickness, villiform structures, and "dark" epithelial border. (b) the vessel architecture shows tortuous and dilated capillaries.

to differentiate between neoplastic and nonneoplastic tissue. By using this diagnostic approach, Kiesslich et al. detected 4.75 times more dysplastic lesions than with conventional endoscopy. There was a reduction in the number of biopsy specimens by half. In addition, CLE was able to predict neoplastic changes with an accuracy of 97.8% [13].

A pilot study on 22 patients of van den Broek investigated the feasibility and diagnostic accuracy of CLE in conjunction with NBI high definition endoscopy in surveillance of patients with long-standing UC [10].

They reported a diagnostic accuracy of 81%, with a moderate interobserver agreement, reflecting their minimal experience with this technique, that certainly needs a learning curve to obtain the best results. They also reported an additional time to colonoscopy of 30–40 minutes to capture images that were evaluated in a second time, and video image quality was than rated as good–excellent only in 69%. For sure, the time needed to perform CLE and to use it to have real-time evaluation of colonic mucosa and taking on-table decision is a limit to the general application of CLE in clinical practice.

Hurlstone et al. [2] assessed the clinical applicability and predictive power of the CLE for the in vivo differentiation of ALM and DALM in CUC. The in vivo diagnosis of DALM and ALM using CLE matched the histological evaluation, with a kappa coefficient of 0.91 and an accuracy of 97%. The study evidenced that ALM and DALM can be differentiated with a high overall accuracy, enabling the safe selection of patients suitable for endoluminal resection versus immediate referral for surgery. In a recent case report of our group, pCLE has been used to characterize a DALM in a long-standing UC with high correlation between CLE and standard histopathological examination [14].

5. Conclusions

CLE is a new technique that promises to be an important imaging tool in the management of patients with UC; it can be used to assess and score IBD activity and to monitor response to therapy and it has the potential to allow the developing of new activity markers, without the need for histological confirmation.

It can avoid unuseful biopsy, precisely target biopsy on suspected area, and allow on-table management decision in surveillance.

This technology is best used in conjunction with other "red-flag" techniques and thus can be used for classification of tissue at a site already detected by standard or enhanced endoscopy. Ideally a red-flag technique such as chromoendoscopy should be used to screen the mucosa for areas of interest, to examine with CLE for a histological diagnosis.

The joining of the two techniques could lead to a higher diagnostic accuracy in surveillance endoscopy in UC patients and to the detection of neoplasia at an earlier and curative stage.

There are some limitations to the application in general practice: the need for a learning curve, the cost of the equipment the need for an extra time to enhanced colonoscopy, and the promising results in the literature being derived from still a few experienced centers. New multicenter studies are needed to assess the cost effectiveness of this technique for surveillance endoscopy in UC.

CLE is a new technology, with the potential, with appropriate training and careful patient selection, to become an important imaging modality in the complex clinical scenario of UC cancer and dysplasia surveillance.

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Review Article

Confocal Endomicroscopy of Colorectal Polyps

Vivian M. Ussui and Michael B. Wallace

Department of Gastroenterology, Mayo Clinic, 4500 San Pablo Road, Jacksonville, FL 32224, USA

Correspondence should be addressed to Michael B. Wallace, wallace.michael@mayo.edu

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Confocal laser endomicroscopy (CLE) is one of several novel methods that provide real-time, high-resolution imaging at a micron scale via endoscopes. CLE has the potential to be a disruptive technology in that it can change the current algorithms that depend on biopsy to perform surveillance of high-risk conditions. Furthermore, it allows on-table decision making that has the potential to guide therapy in real time and reduce the need for repeated procedures. CLE and related technologies are often termed "virtual biopsy" as they simulate the images seen in traditional histology. However, the imaging of living tissue allows more than just pragmatic convenience; it also allows imaging of living tissue such as active capillary circulation, cellular death, and vascular and endothelial translocation, thus extending beyond what is capable in traditional biopsy. Immediate potential applications of CLE are to guide biopsy sampling in Barrett's esophagus and inflammatory bowel disease surveillance, evaluation of colorectal polyps, and intraductal imaging of the pancreas and bile duct. Data on these applications is rapidly emerging, and more is needed to clearly demonstrate the optimal applications of CLE. In this paper, we will focus on the role of CLE as applied to colorectal polyps detected during colonoscopy.

1. Purpose of Paper

The purpose of the paper is to assess the importance of probe-based confocal endomicroscopy on colorectal polyps with a particular emphasis on distinguishing hyperplastic from neoplastic polyps.

2. Recent Findings

New endoscopic imaging modalities have emerged within the past few years and have created a new concept towards diagnosis. Recently, we have seen the evolution of endoscopy from high-definition white light endoscopy and color-enhancement methods, to the novel electronic optical technology known as "virtual biopsy," with increased magnification now approaching that of light microscopy. Color-enhancement methods include chromoendoscopy with topical dyes, narrow-band imaging (NBI), autofluorescence endoscopy, Raman spectroscopy probes, and trimodal spectroscopy [1].

Also, Fujinon intelligent color enhancement (FICE) and iScan (Pentax) are examples of computed virtualchromoendoscopy imaging, both different from NBI, which do not depend on optical filters [2].

Confocal laser endomicroscopy (CLE) allows real-time imaging of the GI tract at approximately 1000-fold magnification with resolution of approximately 1 micron. At this level, visualization of mucosa and lamina propria, as well as single cells is achievable with real-time acquisition speeds [3, 4].

A standard classification system has been developed for both eCLE, termed the Mainz classification [3], and for pCLE, termed the "Miami" classification [5]. These systems distinguished neoplastic from hyperplastic polyps of the colon based on a dark, irregularly thickened epithelial layer characteristic of epithelial dysplasia.

3. Introduction

Colorectal cancer (CRC) is the fourth most commonly diagnosed cancer among men and women and ranks as

the second most common cause of death from cancer in the United States [6, 7].

The importance of colonoscopy with polypectomy to reduce colorectal mortality is well known [8].

The primary purpose of colonoscopy is to identify precancerous polyps and remove them before they become malignant. Extensive studies have identified adenomatous polyps (and some serrated polyps) as the primary precursor of colorectal cancer. Other polyps such as small hyperplastic polyps especially those in the distal colon have little malignant potential. A fundamental limitation of colonoscopy is that, until recently, the only method to reliably diagnose adenomatous and hyperplastic polyp was to remove them and examine them histologically. This reliance on histology implies that there is an undesirable cost and a small risk of removing polyps with little neoplastic risk.

Hyperplastic polyps represent around one-third to one-half of all the polyps [9, 10]. Also of importance are the serrated adenomas. These types of adenomas are manifested in two varieties: traditional serrated adenoma typically assuming a polypoid appearance and the sessile serrated adenoma, flat or slightly raised and located on the proximal colon. This type of adenoma presents some molecular features different from the other colon adenomas and might develop a malignant presentation through a so-called "serrated neoplasia pathway [11]."

Although the absolute risk of polypectomy is small, it is still the most important cause of complication of colonoscopy [12]. Because of the increasing ability to distinguish hyperplastic and neoplastic polyps in vivo, as well as the increasing cost of pathologic examination of all polyps, the concept of using in vivo diagnosis to direct polypectomy has emerged. There are two potential applications of this: first, direct polypectomy only to neoplastic (and serrated) polyps and leave small, low-risk hyperplastic polyps in situ. The second application, termed "diagnose and discard," relies on in vivo diagnosis of low-risk adenomas followed by resection without further histology.

Considering the information above about colon polyp characteristics with one-third to one-half of these polyps being hyperplastic, smaller than 10 mm, and with very low likelihood of malignancy potential [10], selecting the right polyps to remove is of utmost importance. This has the potential to reduce the risk of complications, eliminate unnecessary costs for histopathology analysis, and save time when making a decision for the patient's treatment. Several groups, including the American Society for Gastrointestinal Endoscopy (ASGE), have provided guidelines for when it would be clinically acceptable to adopt a virtual biopsy approach. They set clear thresholds for the accuracy that must be achieved (at least 90% negative predictive value for adenomatous polyps and at least 90% accuracy for predicting the correct surveillance interval). They also set clear target lesions (small distal polyps) that are both the most common polyps as well as those at lowest risk of malignant degeneration in the unlikely event that an incorrect diagnosis is made [13].

Many advanced endoscopic imaging techniques, including dye-based chromoendoscopy and digital chromoendoscopy, have been carefully studied with these goals in mind.

Some impractical aspects of dye-based chromoendoscopy, such as longer procedure times, different dyestainings, and washing techniques, contributed to its limited application. Although these factors do not affect the "virtual" chromoendoscopy methods, such as narrow-band imaging (NBI) or Fujinon intelligent colon enhancement (FICE), an extensive review by the ASGE on these methods shows modest and variable accuracy [13]. CLE has also been extensively studied with regard to colorectal polyps [3, 4].

4. Confocal Systems/Methods

There are currently two clinically available CLE systems; one that is integrated into a Pentax endoscope (eCLE) with the confocal imaging window located at the distal tip of the endoscope. This system allows resolution of approximately 0.8 microns, as well as variable depth of focus from the surface of the lens (0 microns) to a depth of 250 microns by using a control built into the endoscope handle. Still images are obtained at approximately 1 per second.

The second system is based on a through-the-scope probe (pCLE), produced by Mauna Kea Technologies. With pCLE, the laser scanning unit is mounted outside the endoscope, and a bundle of optical fiber, approximately 2.5 mm in diameter, delivers light and collects the images. Proprietary algorithms correct for image distortion of the long fiber bundle. The pCLE system has slightly less resolution (1 micron) and a fixed imaging depth (50 microns); but allows for faster video-rate scanning (12 frames per second) and a far greater versatility with any endoscopic system, including cholangioscopy, through the needle probes for intratumor/intracystic imaging and other non-GI endoscopes (cystoscopy, bronchoscopy, etc.).

4.1. Clinical Image Acquisition. All CLE imaging systems are optimized by a contrast agent, such as fluorescein sodium, discussed in detail below. The quality of the image is affected by several technical factors such as the timing of injection, probe position, and probe stability.

The optimal timing of imaging is obtained within the first 8–10 minutes after contrast injection, although acceptable imaging can be obtained for up to 60 minutes after injection [14]. In order to minimize tissue and vascular leak artifact, the probe should be placed gently in direct contact with the tissue without pressure or trauma.

The probe should be as perpendicular to the mucosa as possible. This can be challenging in the esophagus. Maintaining probe stability is critical to good image acquisition. With the eCLE system, suction is applied to the tissue immediately adjacent to the target lesion to hold the image steady. With pCLE, using the free-hand method can be challenging, but is facilitated by use of a clear 4 mm cap on the tip of the endoscope with slight suction, which can help keep the probe in the proper site.

Table 1: Colorectal pathology prediction using confocal pattern classification.

Grade	Vessel architecture	Crypt architecture
Normal	Hexagonal, honeycomb appearance	Regular luminal openings, homogenous layer of epithelial cells
Regeneration	Hexagonal, honeycomb appearance with no or mild increase in the number of capillaries	Star-shaped luminal crypt openings or focal aggregation of regular-shaped crypts with a regular or reduced amount of goblet cells
Neoplasia	Dilated and distorted vessels; irregular architecture with little or no orientation to adjunct tissue	Ridged-lined irregular epithelial layer with loss of crypts and goblet cells; irregular cell architecture with little or no mucin

Table 2: Systematic classification of colorectal lesions (eCLE based).

	General architecture	Cytonuclear features
Normal mucosa	Regular (uniform) architecture of surface and glandular epithelium	Epithelial cells are uniformly lined up along the basement membrane
	Regular "honey-comb" appearance of vascular pattern	Normal cell polarity of surface and glandular epithelium, normal aspect of mucin-producing goblet cells
	Slightly disturbed architecture: enlarged, branch-like, elongated crypts	Epithelial cells are morphologically normal, preserved cell polarity
Nonadenomatous polyps	Increased number of cells in the crypts	Depletion of goblet cells
	Mild alterations of vascular pattern	
	Inflammatory infiltrate of lamina propria, decreased crypt/stroma ratio	
Adenomatous polyps	Disturbed architecture: mild irregularity of the crypts, eventual villous transformation, increased crypt/stroma ratio, crypt destruction	Incomplete to lack of epithelial surface maturation
	Mild to moderate alterations of vascular pattern	Slightly cytonuclear atypia
		Islands of malignant cells

Previously published studies with eCLE have used topical acriflavine dye, but its use has been diminished as a consequence of the possibility of damage to the DNA cells. Alternately, fluorescein, a relatively inexpensive and safe contrast agent already approved by the FDA for diagnostic angiography and angioscopy of the retina, has been commonly used during pCLE procedures. It can be used topically and intravenously with an excellent safety profile [15].

One to five mL intravenous injection of a 10% solution enables the visualization of each cell, intensely contrasting the capillary network [14, 16, 17]. The initial images in the first 10–30 seconds are predominantly vascular; however as fluorescein leaks into the extravascular space, epithelium and stromal tissues are visualized. Neoplasia tissue appears as dark epithelial cells, which could be explained by the lack of fluorescein absorption, accelerated expulsion from the cell, or greater leakage into the lamina propria [5].

The lack of direct nuclear visualization does not allow the comparison between nucleus and cytoplasm and, therefore, cannot be used for diagnosis and grading intraepithelial malignancies [18]. However, the contrast of the dark color of the neoplastic epithelium permits architectural analysis of the surface mucosa and aids in differentiating normal mucosa from neoplastic tissue [19].

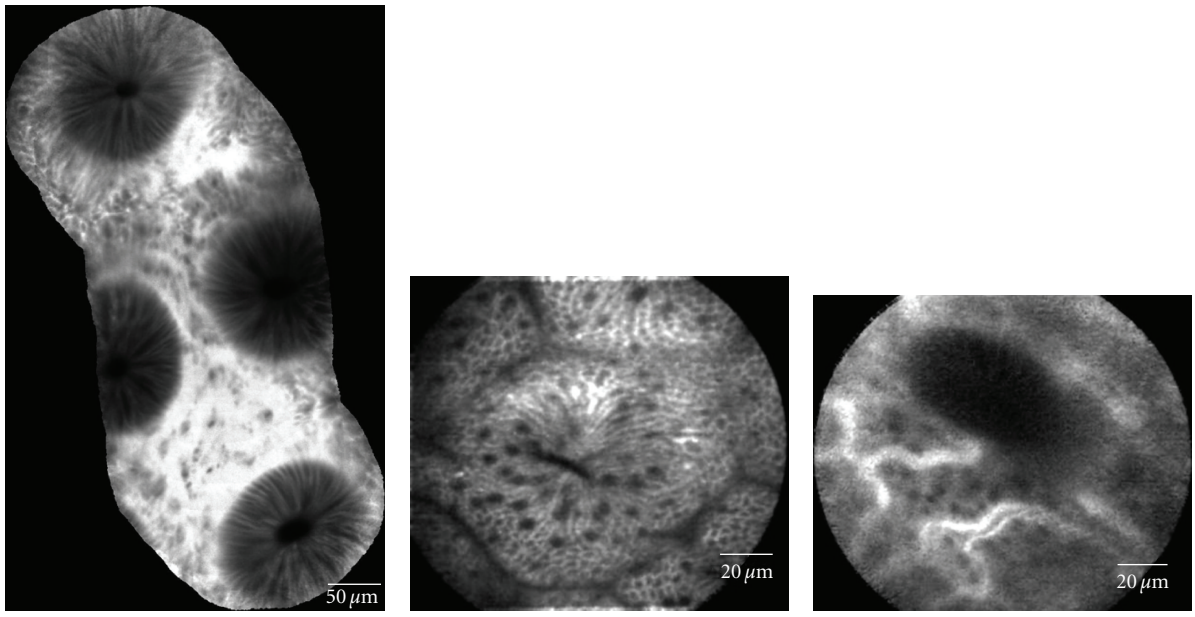
The major side effects of fluorescein are temporary, lasting up to 24 hours and consist of skin discoloration and a yellowish tint to the urine. Other complications include nausea, hypotension, and mild skin rash. Serious complications, such as anaphylaxis or infection site reactions, are extremely rare [15].

Table 3: Potential applications of pCLE or CLE.

Areas that have been well evaluated	Barrett's esophagus guide to biopsy
	Colon polyp classification
Areas of early exploration	Inflammatory bowel disease—dysplasia
	Biliary strictures
	Duodenal neoplasia
Experimental areas	Solid and cystic tumor imaging
	Gastric neoplasia

4.2. Grading Confocal Images. The Mainz criteria, developed by Kiesslich et al. [3] in 2004, described patterns of normal, regenerative, and neoplastic tissue as seen by eCLE (Table 1). Sanduleanu et al. [20] further defined the key patterns in colorectal polyps (Table 2).

Finally, the Miami classification system, which is similar to the Mainz system, was developed specifically for pCLE images (Table 3) [5]. Examples of normal colonic tissue are



(a) (b) (c)

Figure 1: Normal colon epithelium. (a) Mosaic image of normal crypts, which are circular and evenly distributed. Although the epithelium is relatively dark compared to the bright stroma, the thickness is very regular with uniform crypt structures. (b) Single crypt opening, slightly slit-like but with abundant goblet structures (dark "dots" within the epithelial cells). (c) Capillary vasculature typically seen very early in the injection. The vessels are small (<10 mm) in diameter and form a regular capillary network.

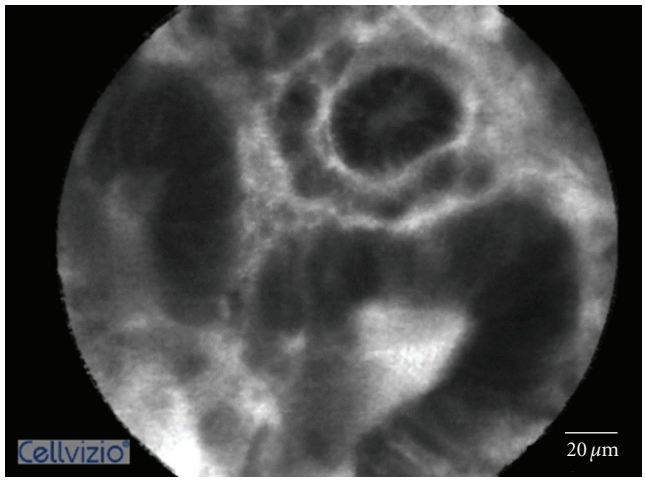


Figure 2: Adenomatous polyps. The epithelium is much darker with irregular thickness and forming small villous-like structures with wide openings.

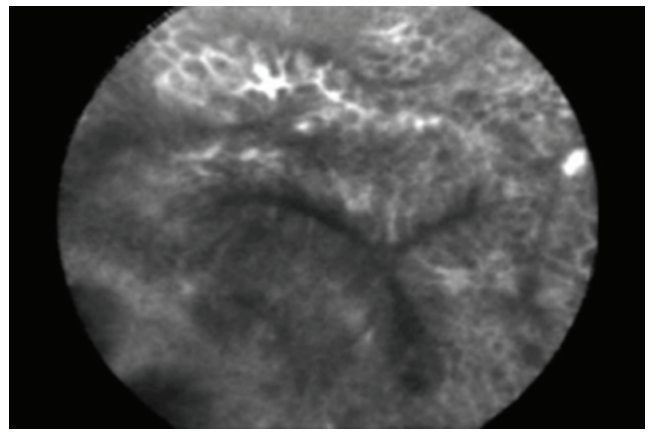


Figure 3: Hyperplastic polyp. The crypt opening is stellate in shape similar to Kudo type 2 pit patterns. The epithelium is relatively bright and regular in thickness.

shown in Figures 1(a)–1(c). Examples of colonic adenomas and hyperplastic polyps are shown in Figures 2 and 3.

5. Clinical Application

5.1. Colon Polyps. The following methods have been applied to predict histology based on the Kudo pit pattern, vascular pattern intensity (VPI), and color: Chromoendoscopy (CE), narrow-band imaging (NBI) and a combination of NBI and autofluorescence imaging (AFI) called endoscopic trimodal imaging (ETMI), and CLE. Regarding colon polyps and CLE, the most significant reason for its application is the capability of discerning a hyperplastic from an adenomatous polyp.

The first report of the use of endoscope-based CLE was published by Kiesslich et al. [3]. Their study of 42 patients has proven the ability to predict the presence of neoplastic alterations with a very high degree of accuracy (sensitivity, 97.4%; specificity, 99.4%; accuracy, 99.2%).

De Palma et al. [21] further reported the accuracy and interobserver agreement for pCLE in colorectal polyps. In a study involving 32 small polyps in 20 patients ranging

from 1 to 9 mm, comparing pCLE and NBI, pCLE achieved a sensitivity and negative predictive value of 100% and specificity of 85% in predicting adenomatous histology.

Our group recently reported a comparison of pCLE to virtual chromoendoscopy (NBI or FICE). Sensitivity of pCLE was higher than virtual chromoendoscopy (91% versus 77%) $P = 0.01$ with similar specificity (76% versus 71%). When looking at subgroups of polyps imaged NBI versus FICE, the advantage of pCLE was only seen in comparison to FICE imaging with statistically similar accuracy when compared to NBI [22]. This study included both large (>9 mm) and small polyps. Therefore, our group has further explored the accuracy of pCLE, whereas NBI focused exclusively on the small polyps that may be eligible for a diagnose-and-discard strategy. In this study, pCLE and NBI were evaluated both independently and in combination.

One hundred and thirty polyps <10 mm were evaluated in 65 patients. pCLE had a higher sensitivity than NBI (86% versus 64%, P 0.008) but with lower specificity (78% versus 92%, P 0.027) and similar overall accuracy. When combining pCLE and NBI, limiting the analysis to high-confidence images, the sensitivity and negative predictive value was 94% and specificity 97%. This is important as it demonstrates the technology can exceed the ASGE recommended thresholds (90% or greater) for acceptance of a diagnose-and-discard strategy [23].

The previous trials of both eCLE and pCLE have largely evaluated offline image interpretation, which does not allow on-table decision such as diagnose and discard. Our group has recently compared real-time and offline pCLE interpretations of colorectal lesions. Although real-time interpretation accuracy was slightly lower (78% versus 81%), these differences were statistically equivalent [23, 24].

An article assessing the learning curve of in vivo pCLE for prediction of colorectal neoplasia indicated that a wide range of GI specialists could become proficient in interpreting high-quality pCLE images after review of 50–70 cases and approximately 2 hours of training [9].

6. Conclusion

Confocal laser endomicroscopy has brought several insights and new concepts to the endoscopic field. Considering all the advantages and drawbacks, it is important to highlight a few aspects.

(1) Though the learning curve does not appear to be long, some training is required to achieve a consistently high level of accuracy.

(2) The combination of virtual chromoendoscopy, such as NBI and CLE, has proven to be a highly accurate method for in vivo diagnosis, allowing a careful virtual panchromocolonoscopy followed by target endomicroscopic examination.

(3) pCLE has shown higher sensitivity but similar specificity compared to NBI for small polyps, especially those sized 1–5 mm.

(4) Although promising, the cost of CLE is still high relative to histology. In order to become more clinically relevant, the cost must be reduced through more durable, less expensive probes and integrated endoscopic devices.

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Review Article

Endomicroscopy and Cancer: A New Approach to the Visualization of Neoangiogenesis

Renato Cannizzaro,[1] Maurizio Mongiat,[2] Vincenzo Canzonieri,[3] Mara Fornasarig,[1] Stefania Maiero,[1] Valli De Re,[4] Federico Todaro,[2] Paolo De Paoli,[5] and Paola Spessotto[2]

[1] *Department of Gastroenterology, National Cancer Institute, IRCCS, Via F. Gallini 2, 33081 Aviano, Italy*
[2] *Department of Experimental Oncology, National Cancer Institute, IRCCS, Via F. Gallini 2, 33081 Aviano, Italy*
[3] *Department of Pathology, National Cancer Institute, IRCCS, Via F. Gallini 2, 33081 Aviano, Italy*
[4] *Department of Clinical and Experimental Pharmacology, National Cancer Institute, IRCCS, Via F. Gallini 2, 33081 Aviano, Italy*
[5] *National Cancer Institute, IRCCS, Via F. Gallini 2, 33081 Aviano, Italy*

Correspondence should be addressed to Renato Cannizzaro, rcannizzaro@cro.it

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Probe-based Confocal Laser Endomicroscopy (pCLE) is a novel imaging technique for gastrointestinal endoscopy providing *in vivo* microscopy at subcellular resolution. It offers the possibility to analyze neoangiogenesis and vessel density *in vivo*. Angiogenetic switch is essential in cancer progression. Aim of the paper was to review the use of this imaging tool to analyze colorectal and gastric cancers vascularization *in vivo*. The aim is to provide the possibility of combining diagnostic evidences with vascularization and molecular profile to evaluate the efficacy of an antiangiogenic treatment in association with conventional therapy. pCLE can be considered a revolutionary method for real-time assessment of changes in vascularization pattern in this tumors and it may open the possibility to address the use of anti-angiogenic therapy in order to improve the outcome of the treatment.

1. Introduction

In recent years, endoscopic image quality has been highly improved thanks to very technologically advanced devices. One of these new gastrointestinal endoscopy methods is the confocal laser endomicroscopy (CLE) which has allowed for a confocal scanning microscope to be integrated into a conventional flexible endoscope. Potential applications include detection of neoplasia with targeting of biopsies, and the detection of inflammatory bowel disease, celiac sprue, and microscopic colitis [1]. CLE provides *in vivo* microscopy at subcellular resolution: imaging of the mucosal layer at resolution at approximately 1 micron and visualization of the cellular and subcellular structures as well as capillaries and single red blood cells are peculiar characteristics of this novel gastrointestinal endoscopy method. We experienced with the probe-based confocal endomicroscopy system (pCLE, Cellvizio, Mauna Kea Technology, Paris, France), in combination with videomosaicing which allows the reconstitution of panoramas of the tissues with the alignment of the input frames. We aimed therefore to use this new promising imaging tool to analyze the angiogenic process in colo-rectal and gastric cancer patients. This method discloses the possibility of a translational approach combining the confocal imaging not only with the diagnosis *in vivo* and the specific molecular profile of the patient, but also with the targeted antiangiogenic treatment.

2. Endomicroscopy and Tumors

The potential role of CLE has been explored in different pathologic conditions of the gastrointestinal (GI) tract, the possibility of diagnosing premalignant and malignant lesions of the GI tract being particularly important considering the prognostic implications. GI cancers represent a major cause of morbidity and mortality, with incomplete response to chemotherapy and poor prognosis in the advanced stages of the disease. Recently, CLE has been successfully applied in

studies dedicated in particular to neoplastic Barrett's esophagus, and gastric and colorectal neoplasia. Since accurate diagnosis and staging are essential for therapeutic planning, CLE holds the potential for a strong impact in the screening and/or surveillance of GI tumors [2, 3]. CLE has been used in a pilot study also for detection of biliary malignancy [4]. All the studies performed revealed the clinical usefulness and predictive power for the high-resolution probe-based CLE for *in vivo* diagnosis of GI neoplasia and related precursor lesions during colonscopy. Based on characteristic morphological changes or due to characteristic single cells like globet cells in Barrett's esophagus, the promising technology of CLE enables already *in vivo* diagnosis of pathological mucosal conditions. However, confocal imaging holds the potential to go far beyond: the possibility to analyze the morphology and density of the blood vessels present on the surface of the tumors could also provide vital information for a more appropriate diagnosis and for a putative employment anti-angiogenic drugs during the treatment.

3. Angiogenesis Markers

The development of new blood vessels from the preexisting vasculature (angiogenesis) is an indispensable event both in normal and pathological conditions, such as cancer growth and development. Tumors will not grow beyond 1-2 mm unless the angiogenic switch is turned on [5], thus the formation of novel blood vessels is regarded as one of the most important events occurring in the neoplastic process [6]. In fact, the development of new vessels supplies the growing tumor with nutrients and oxygen, disposing metabolites and releasing growth factors that promote tumor cell proliferation [7]. Indeed tumors promote angiogenesis by secreting growth factors such as vascular endothelial growth factor (VEGF), hepatocytes growth factor, and platelet-derived growth factor that stimulate endothelial migration and proliferation [7–9]. The binding of VEGF to VEGFR triggers an intracellular signaling that is mainly mediated by MAPK and PI3K/Akt/mTOR pathways. This results in the expression of HIF-1a and induction of PDGF, FGF, G-CSF, TGFβ, and angiopoietins, thus enhancing angiogenesis [10]. Angiogenesis is also indispensable to the metastatic process by providing large numbers of leaking blood vessels for vascular invasion [7]. To early detect and assess the extent of the intratumoral angiogenesis is thus crucial for a personalized and prompt antiangiogenic therapy. Besides the commonly used panendothelial markers such as CD31, CD34, Factor VIII, endoglin (CD105) has been proposed as a marker of tumor angiogenesis since the endoglin antibody binds preferentially to the activated endothelial cells that participate neovascularization [11, 12]. Endoglin is a receptor for the TGF-β1 molecule and was indeed found to be upregulated during neoangiogenesis [13]. Among the molecules specifically located along the blood vessels, MULTIMERIN2 (a.k.a. EndoGlyx-1) can be considered a good marker for the blood vessels. MULTIMERIN2 (MMRN2) belongs to the (EMI Domain ENdowed) EDEN protein family [14]. The protein was discovered during the search of novel markers of the vascular endothelium [15]. MMRN2 is in association with a high-molecular weight glycoprotein complex. Two subunits of this complex match the MMRN2 sequence and are likely the result of to posttranslational modifications. The other two subunits have not yet been identified [16]. MMRN2 can be considered a panendothelial marker being expressed both in normal and tumoral vasculature including hot spots of neovascularisation in some tumors [15–18]. The molecule was shown to be specifically deposited along the blood vessels in tight juxtaposition with endothelial cells and to be also present in the luminal side of the vessels [16]. Prior to our studies, its function though has remained obscure. Recent data collected in our laboratory [19] indicate that this molecule plays an important role in the regulation of endothelial cell function, tumor angiogenesis, and vessel homeostasis. To carry out these studies, we have developed both antihuman and antimouse antibodies against this molecule which were found to detect blood vessels in many tissue and tumor sections as well as the anti-CD31 antibody [19]. The use of this antibody was found to be suitable for the detection of blood vessels both in colorectal and gastric cancer sections as indicated by our results. Interestingly, MMRN2 was also demonstrated to play an important role in vessels maturation (in terms of pericyte coverage) and to regulate also endothelial cell permeability [19]. Thus this molecule may not only be a mere marker of blood vessels but also be important, depending on its expression, in predicting the vessel functionality. Indeed, the combination of the MMRN2 staining with the pCLE analysis could provide a more reliable evaluation of the "angiogenetic status" of the patients.

4. Endomicroscopy and Angiogenesis

High-resolution confocal imaging is achieved by using an exogenous fluorescence technique. Fluorescein is intravenously administered for specific *in vivo* imaging of human colorectal neoplasia and its use also allows the analysis of the vascular structure, morphology (irregular vessels) and leakiness (fluorescein outflow). Studies are currently underway to apply this new imaging tool for objective evaluation of the microvessel density in different stages of the neoplastic development and in conjunction with antiangiogenetic therapy. Preliminary data on the microvessel density for biliary cancers at the liver hilum [20, 21], for Barrett's esophagus [22], and for GI tumors [23, 24], are currently available.

Endoscopic imaging and monitoring of angiogenesis have the potential to be valuable biomarkers in preneoplastic, premalignant, and cancer stage in GI lesions. The "endoscopic angiogenesis" analysis on gastric and colo-rectal cancers was performed on the patients listed in Table 1. We evaluated pCLE images from 25 sequences/biopsy sites and compared with the histological data. The vascular architecture in cancer patients was abnormal (enlarged, tortuous microvessels with altered blood flow). The morphological pattern of neoangiogenesis was in accordance with the histology and immunohistochemical analysis, allowing us to develop an arbitrary "angiogenesis" scale whose criteria are reported in Table 2. The Cannizzaro-Spessotto scale evaluates the extent of intratumoral angiogenesis based on the increase of the number of vessels, the presence of tortuous and large vessels,

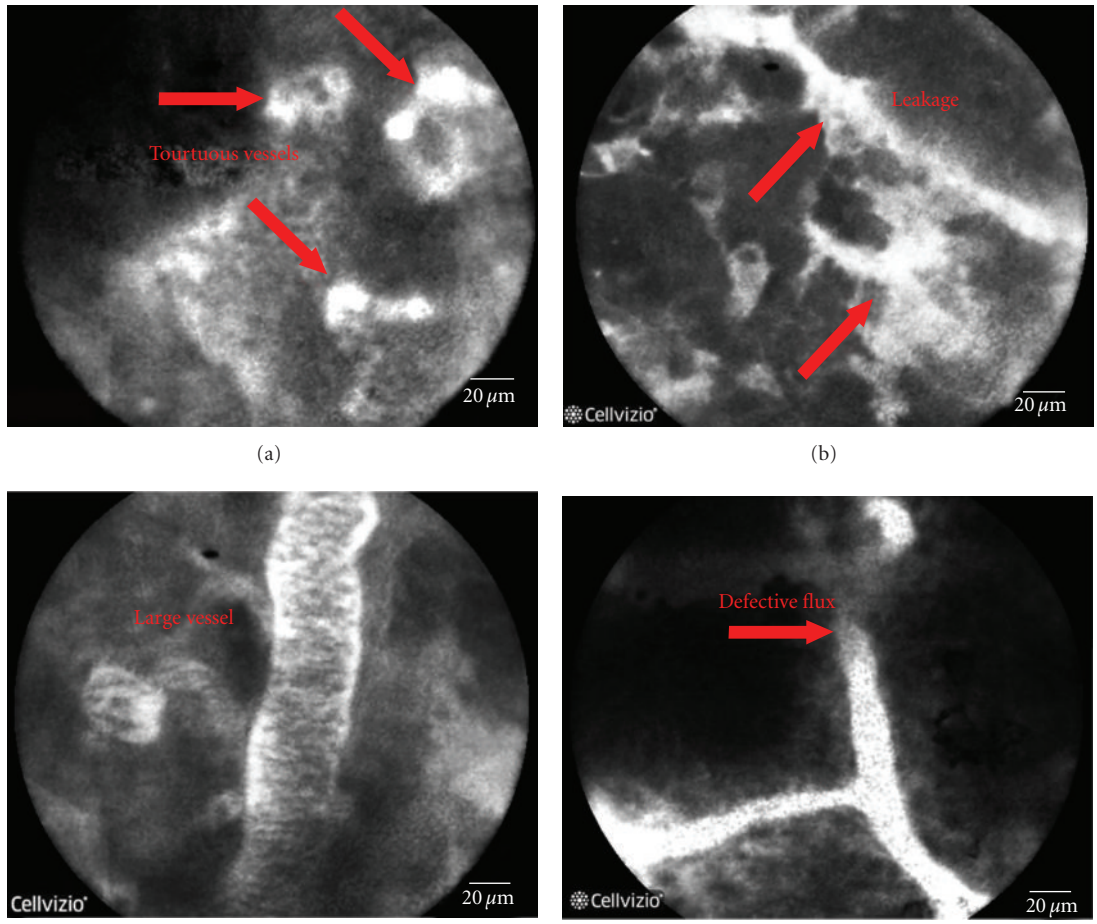

Figure 1: Representative pictures of the images obtained during pCLE endoscopy where the presence of tortuouse large blood vessels with defective flux and leakage are indicated by the arrows.

Table 1: Patients enrolled for pCLE analysis.

Patient	Age	F/M	Diagnosis	Grade/Staging (EUS)
1 DM	64	M	Adenoma	
2 ML	62	F	Rectal neoplasia	T3N+
3 GG	60	M	Gastric neoplasia	T1N0
4 CG	51	F	Rectal neoplasia	T3N+
5 ZI	71	M	Rectal neoplasia	T3M+
6 CP	63	F	Rectal neoplasia (stenosis)	
7 GM	77	F	Rectal neoplasia	T3N0
8 SS	38	M	Rectal neoplasia	T3N + M?
9 TB	68	M	Rectal neoplasia	T3Nx
10 TC	54	M	Rectal neoplasia	T3N+
11 FL	66	F	Gastric neoplasia	T3N+

Table 2: Calculation of Cannizzaro-Spessotto scale.

Criterion	Score	
	No	Yes
Tortuous vessels	0	1
Large vessels	0	1
Leakage	0	1
Defective flux	0	1

The patient' s angiogenesis index is obtained by summing the scores of the single items.

fluorescein leakage, and defective flux (Figure 1). Even if preliminary, these data (reported in Table 3) suggest that the application of Cannizzaro-Spessotto scale could be helpful in predicting the response to anti-angiogenic therapy and possible chemoresistance of a tumor during treatment and if the treatment received has been insufficient to avoid surgery. Further data on a greater number of tumors at different stages are needed to improve the diagnostic accuracy and to guide and predict the more appropriate individualized strategies for the treatment.

Table 3: "Angiogenetic status" of the patients.

Patient	"Angiogenesis" index (Cannizzaro-Spessotto scale)
1 DM	0
2 ML	3
3 GG	2
4 CG	2
5 ZI	Not applicable (stenosis)
6 CP	Not applicable (stenosis)
7 GM	4
8 SS	3
9 TB	2
10 TC	2
11 FL	3

5. Conclusions and Perspectives

The use of pCLE allowed the *in vivo* assessment of the morphological alterations and the abnormal microvasculature of the cancer mucosa and the results correlated with the traditional conventional approach. pCLE can be considered a crucial and revolutionary method for real-time analysis of the vascularization pattern in colo-rectal and gastric cancer. These preliminary results indicate that pCLE in combination with immunological staining (CD31 and MMRN2) hold the potential for a significant impact both on basic research and clinical practice, suggesting a substantial possibility for a translational study.

Disclosures

R. Cannizzaro and M. Mongiat shared first authorship.

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