Identification of Bronchoalveolar Lavage Components Applying Confocal Laser Endomicroscopy

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Background: In many studies, confocal laser endomicroscopy (CLE) has proven to be a useful tool in pulmonology; nevertheless, the application in this field is still experimental. By contrast, CLE is almost a standard technique in gastroenterology. The aim of the present study was to demonstrate the identification of bronchoalveolar lavage (BAL) components applying CLE, using a dye.

Material/Methods: In 21 patients with various underlying diseases a bronchoscopy with BAL was performed. As in routine clinical practice common, BAL fluid (BALF) was analyzed in terms of cytologic, virologic, and microbiologic aspects. To one fraction of BALF, we added acriflavine. After centrifugation CLE was applied and the video sequences were analyzed by an experienced investigator.

Results: Using CLE, BALF components (such as alveolar macrophages or leucocytes) could be easily identified. A further subdivision of leucocytes (neutrophilic, eosinophilic granulocytes, and lymphocytes) was not possible. Analogous to conventional cytology, a precise distinction of lymphocyte subpopulation (cd 4/cd 8 ratio) was not feasible. In terms of quantification, this is still the application field of flow cytometry and immunohistochemistry.

Conclusions: Using CLE, alveolar macrophages and leucocytes in stained BALF can be differentiated independent of smoking status. Further studies should be initiated in order to subclassify leucocytes in eosinophilic, neutrophilic granulocytes, and lymphocytes, which is important for routine clinical practice.

MeSH Keywords: **Acriflavine • Bronchoalveolar Lavage Fluid • Microscopy, Confocal**

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Background

Confocal laser endomicroscopy (CLE) is a widely used technique in gastroenterology. It is an endoscopic tool that allows *in vivo* (during ongoing endoscopy) or *in vitro* histology at subcellular resolution by using (fluorescence) contrast agents [1]. The most commonly used contrast agents are acriflavine hydrochloride (topical use only) and fluorescein sodium [2].

In gastroenterology, CLE has been used for detection, classification and/or monitoring of colon polyps/cancer [3], Barrett's esophagus [4], ulcerative colitis, Crohn's disease [5], and many other conditions. In contrast, in pulmonology this method lacks a well-recognized scope. However, Thiberville et al. were able to display parts of normal proximal and distal bronchial system during a flexible bronchoscopy with CLE [6]. Furthermore, CLE has been effectively used for diagnosis and monitoring of pulmonary alveolar proteinosis [7,8], as well as for diagnosis of pneumocystis jirovecii and amiodaron related pneumonia [9,10]. Likewise, characteristic changes in cases of acute lung allograft rejection have been identified [11]. Other encouraging studies have been performed showing the differentiation of normal bronchial mucosa from cancer tissue *in vivo* and *ex vivo* using CLE [12,13]. Even solitary lung nodules have been imaged applying CLE [14,15]. Nevertheless, CLE is still not implemented in routine clinical practice and the application currently is thought to be experimental.

In quest of an appropriate area of application, our study group analyzed CLE-based pleural effusions. CLE permitted simple and rapid detection of malignant cells in previously stained pleural effusions (data not shown, publication in progress). Furthermore, this study was initiated to determine the value of CLE in characterization of BALF components. In previous studies, BALF underwent already a CLE-based analysis but only in a native setting. When consistently used in present or former smokers, alveolar macrophages as the main BALF component have been shown due to tobacco tar specific fluorescence [16]. This effect holds even after smoking cessation for a long time (for a minimum of 6–15 months after smoking cessation with great individual variation) [17]. Sometimes the fluorescence imparted upon alveolar macrophages from cigarette smoking can take years to fully diminish [16,17] and there is *in vitro* evidence that air pollution has a similar effect on macrophage fluorescence [18]. By contrast, alveolar macrophages were not visible in non-smoking healthy subjects. The same observation was made by performing CLE during bronchoscopy and pushing the probe into the subdivisions of the bronchial tree [10,19]. Interestingly, in some diseases, macrophages and presumably even leucocytes could be detected using CLE natively [10]. For comparable results, and in expectation of better contrast and a more detailed view, we chose to stain the BAL fluid with the often-used dye acriflavine. Previously, we

conducted a pre-pilot study with a small sample size (n=10; 8 smokers, 2 non-smoker) comparing stained to non-stained fluids. With acriflavine, we got a much better contrast. Alveolar macrophages were easily detectable regardless of smoking or non-smoking probands, as well as leucocytes. In unstained samples, sometimes only a non-distinguishable cell mass could be shown by CLE. Therefore, only stained samples were analyzed in this study. To our knowledge, this is the first report on applying CLE to analyze stained BALF.

Bronchoscopic evaluation with BAL has been an approved technique for a long time and provides important diagnostic information in patients with pulmonary diseases (immunodeficient or not) [20]. As a safe method, it is routinely performed when clinically indicated. Such indications might be: microbial detection, differentiation of interstitial pulmonary diseases, or proof of malignant cells. Especially in patients with suspected interstitial lung diseases, a clear and quantitative differentiation of all BALF components (such as alveolar macrophages, lymphocytes, neutrophilic or eosinophilic granulocytes) is pivotal for diagnostic and therapeutic decisions. The aim of this study was to evaluate whether CLE-based analyses can provide this important information "real-time".

Material and Methods

This prospective study was approved by the local ethical committee (*http://www.ethikkomitee.med.uni-erlangen.de*) and was conducted according to the Declaration of Helsinki. Patients who underwent bronchoscopy with bronchoalveolar lavage between August 2012 and September 2012 for evaluation of pulmonary infiltrates were recruited for this study. The bronchoscopic procedure was explained to all participants in detail and participants gave their written informed consent. Twenty-one patients suffering from diverse underlying diseases were included in this study (Table 1). All participants underwent a flexible white light video-chip bronchoscopy (Olympus BF 1T180 or BF Q180, Olympus, Tokyo, Japan). For sedoanalgesia, we used intravenous midazolam (5 mg to 8 mg) alone or in combination with pethidine (50 mg to 100 mg). For topical anesthesia we used lidocaine solution limited to 4.5 mg/kg body weight. For routine monitoring, ECG, pulse oximetry, and intermitted non-invasive measurement of blood pressure were reported. Each patient received a minimum of 2 L O_2/minute via nasal probe.

First the bronchial system was carefully inspected. Anomalies and signs of malignancy were documented in detail and biopsied after realization of BAL.

BAL was performed with 100 mL lukewarm sodium chloride 0.9% solution at the radiological suspicious lung area. The

Table 1. Patients characteristics.

Patient No	Age years	Sex	Smoking	Histology/cytology	Underlying disease	Detection*
1	59	M	Ex, 40PY	Lymphoma infiltration	Lymphoma	Mainly leucocytes
2	70	M	Ex, 40PY	Regular	Suspected pneumonia	Regular
3	70	M	Ex, 40PY	Regular	Suspected pneumonia	Regular
4	25	M	Yes, 2–3 cig./d	Regular	Suspected tbc	Regular
5	66	F	Ex, 30PY	Inflammatory, regular	NSCLC	Regular
6	56	M	Yes	30% lymph	HIV	Increased leucocytes
7	50	M	Ex for 7y	Regular	Sarcoidosis	Regular
8	51	F	Yes	Regular	SCLC	Regular
9	46	M	No	Inflammatory	Lung adenocarcinoma	Increased leucocytes
10	49	M	Yes, 20 cig./d	Regular	Mycosis fungoides	Regular
11	28	M	No	Regular	Suspected tbc	Regular
12	82	F	No	Inflammatory	Suspected eaa	Increased leucocytes
13	47	F	Yes, 15PY	Inflammatory, regular	COPD	Regular
14	21	M	Yes	Regular	Osteosarcoma	Regular
15	50	M	Yes	Regular	Parotid cancer	Regular
16	67	M	No	Inflammatory	IgG 4 related disease	Increased leucocytes
17	33	F	Ex, 5PY	Inflammatory	ALL	Increased leucocytes
18	67	M	Yes, 50PY	Regular	COPD	Regular
19	78	F	No	Inflammatory	Lung adenocarcinoma	Increased leucocytes
20	63	M	Yes	20% lymph	Dyspnea	Regular, incr. leuco.
21	31	M	No	50%lymph	Sarcoidosis	Regular

* Regular=84–99% alveolar macrophages, <1% epithelial cells, <4% neutrophils, <1% eosinophils, <16% lymphocytes; * reactive/inflammatory=increased neutrophils.

recovery fluid (30–50 mL) was divided into 3 or 4 parts. One was sent to microbiology, one to cytology, one in a few cases to virology, and one was used for this study. Acriflavine (2.5 mg acriflavine dissolved in 1 mL sodium chloride 0.9%, Sigma Aldrich) was added to each study sample.

Acriflavine passes through cell membranes and displays a strong specificity for labeling acidic constituents providing a clear visualization of the nuclei and cytoplasm [21]. After shaking the probe carefully (for a good staining result) they were centrifugated (4°C, 1500 rpm, 10 minutes). The cell pellet was than analyzed by CLE by an experienced investigator.

We used the commercial Cellvizio system (Mauna Kea Technologies, Paris, France). A confocal miniprobe S type (1.4-mm diameter; SN: DM-2023, Mauna Kea Technologies)

was applied which displayed a penetration depth of 0–50 µm, a lateral resolution of 3.5 µm and a field of view of 600×500 µm.

The video sequences were assessed with the included software (Cellvizio viewer, version 1.4.1; Mauna Kea Technologies). We selected a grey scaled imaging with the lower and upper level thresholds of the reference lookup table from 0 to 8000 units. The video sequences were recorded for further adjustment.

Results

Twenty-one patients with various infectious, malignant, autoimmune, and other diseases were included in this study (Table 1). Confocal laser endomicroscopy was used as a new diagnostic approach for analyzing BALF. Acriflavine was applied
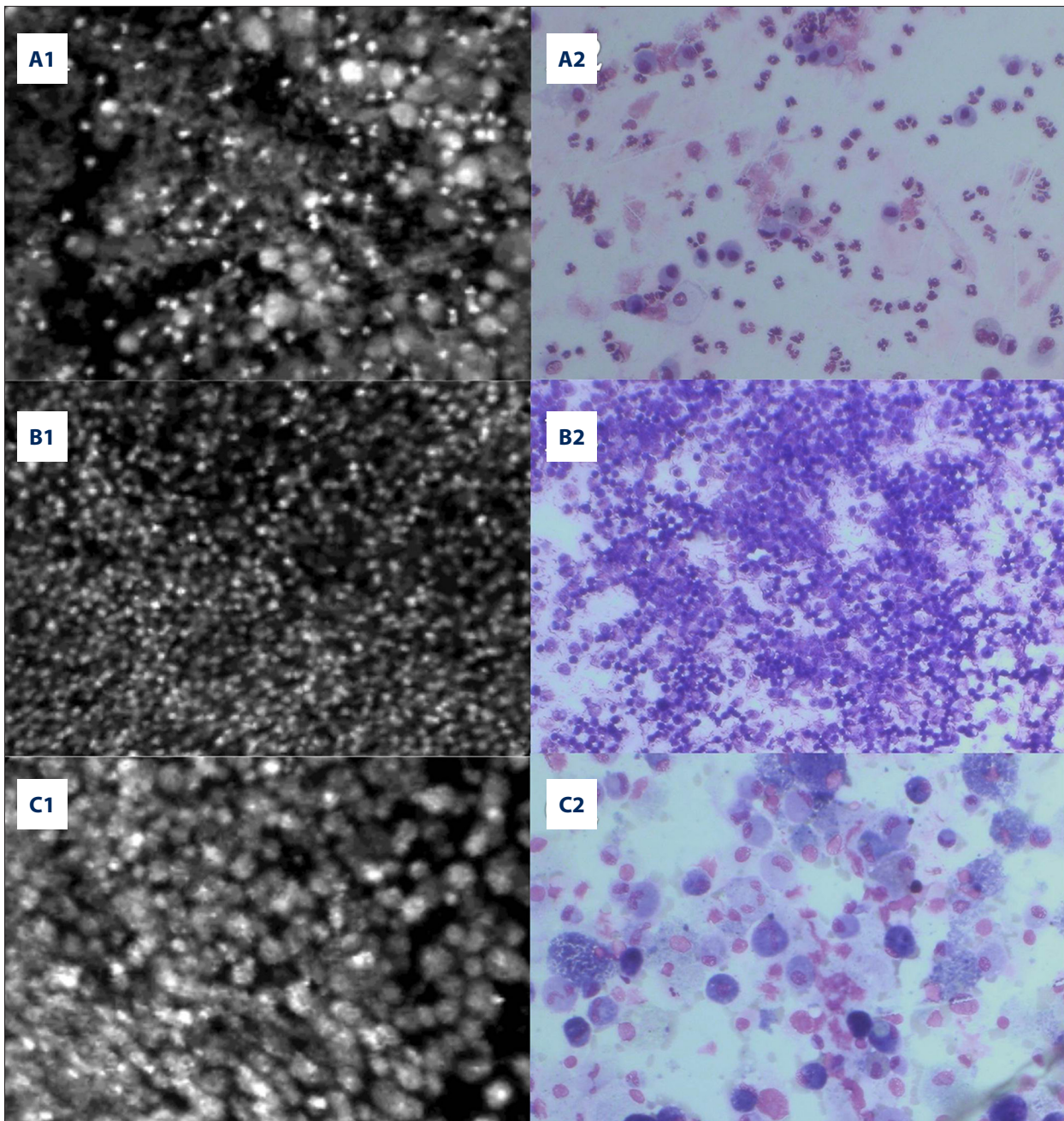
Figure 1. Comparison CLE and cytology. (**A1**) Probe-based confocal laser endomicroscopy with proof of alveolar macrophages and increased leucocytes. (**A2**) Cytological analysis, detection of increased neutrophils corresponding to inflammation. (**B1**) CLE aided detection of predominantly leucocytes. (**B2**) Cytological confirmation of mainly lymphocytes with big cell nuclei, in this case infiltration of high grade lymphoma. (**C1**) CLE: mainly alveolar macrophages, normal result. (**C2**) Cytology: mainly alveolar marcophages and monocytes, normal BALF.

as the contrast agent, predominantly staining cell nuclei and to a lesser extent cytoplasm.

By means of CLE, a good distinction of alveolar macrophages and leucocytes in BALF could be made (Figure 1). A subdivision of primary lymphocyte, eosinophilic or neutrophilic granulocyte predominance was not achievable. Because there were strong cell nuclei staining and a rather small cell-plasma relation, acriflavine resulted in an outshining of the entire cell. So important factors of distinction (nucleus structure: rounded versus more lobular or cytoplasm content) were not visible.

Discussion

Currently, confocal laser endomicroscopy rates high in medicine with evident diagnostic added value. Temporary CLE is especially useful in gastroenterology [3,5,22–31]. In addition, CLE has a possible application in the field of urology [32,33], gynecology [34], neurology [35,36], and ENT medicine [37,38]. Therefore, it is understandable that also in pulmonology several studies on CLE have been initiated. The main point of interest is whether CLE is suitable for pulmonological tissue. Studies have shown possible applications, that are the distinction between normal bronchial mucosa [6,39] and tumorous infiltrated mucosa in *in vivo* and *ex vivo* settings [12,13]. Moreover, pathologically altered tissue within the context of allograft rejection [11], pulmonary alveolar microlithiasis [40], pneumocystis jirovecii pneumonia [9], and amiodarone-related pneumonia [10] could be demonstrated applying CLE. In the past, even solitary lung nodules were classified by *in vivo* imaging using CLE [14,15].

In this study, we focused on the identification of BALF cell components using CLE. This has been done before, but to our knowledge, our study is the first approach using acriflavine-stained BALF CLE-based analysis.

According to other *in vivo* studies [7,19,39], we were able to illustrate alveolar macrophages in BAL fluid. However, these previous findings were restricted to smokers in a native setting due to tobacco tar specific fluorescence [16] (and also to special inflammatory settings such as amiodaron related pneumonia [10] or pulmonary alveolar proteinosis [7]). However, we were able to show alveolar macrophages equally in non-smoking people. A distinction of leucocytes is based on cell size and cell nucleus; however, morphology is also used to easily identify cells (macrophage: 30–50 µm versus neutrophilic granulocyte 12–15 µm versus lymphocyte with varying size 4–20 µm). In an inflammatory setting such as amiodaron-related pneumonia, Salaün et al. reported CLE-based detection of 2 populations of fluorescent cells in the airways of non-smoker without using a special dye. One fraction was considered to represent alveolar macrophages; the other was presumably activated lymphocytes and/or neutrophils [10]. Consistent with these findings, Yserbyt et al. revealed that auto-fluorescent cells were highly present in patients with acute lung allograft rejection [11]. Thus, there might be special circumstances, such as inflammatory cell infiltration and cell activation as a consequence of various diseases, that can lead to an auto-fluorescence of macrophages and leucocytes even in nonsmoking patients [16]. As mentioned before, smoking status has an important influence on macrophage auto-fluorescence. It increases with smoking duration and intensity as well as the number of macrophages; and even after smoking cessation this last for a long time. To avoid these influencing factors and to be able to analyze all samples regardless of disease or smoking status in a comparable

way, requires tested staining methods. In our case, we used acriflavine, known to stain cytoplasm and nuclei. Additionally, this can lead to better contrasted pictures. However, a further clear differentiation of leucocytes and granulocytes (eosinophilic/neutrophilic) and lymphocytes was not part of our study. Since both leucocyte subgroups could present a similar cell size (dependent on the maturation/activation level of the lymphocytes), this method does not provide a reliable distinctive feature. The precise presentability of cell nucleus structure (rounded versus more lobular), cytoplasm content (eosinophilic granules), and nucleus-plasma relation, would be pivotal in this domain. This distinction could not be achieved by the results within the context of our pilot study. The obviously smaller cells in comparison to the alveolar macrophages showed a strong nuclear staining result by means of vital staining with acriflavine. Because of the rather small nucleus-plasma relation, this resulted in an outshining of the entire cell. Therefore, the distinguishing characteristics could not be analyzed. However, a distinction between lymphocytes and granulocytes is essential for routine clinical practice regarding diagnosis and further therapy decision. Thus, a follow-up study to address this issue would certainly be worthwhile. Especially since other research groups have succeeded in this area of study in other medical disciplines. Ji et al. were able to show granulocytes as a predictive marker of helicobacter pylori infection in gastric mucosa [41]. Eventually, a better identification could be achieved by reducing the acriflavine dose for each probe. In the literature, particularly in gastroenterology literature, one can find different recommended data for acriflavine staining dose. The range varies between 0.02–0.05%. There is no detailed statement regarding the "best" dose to use. Recently however, the study group of Li et al. explores this question further [29] and proposed a dose of 0.02%, similar to what was used in our study. Nevertheless, the optimal dose may vary from tissue to tissue or fluid being analyzed. Therefore, a follow-up study should be initiated with a reduced stock solution dose of 0.01%. With a lower dose, the nuclei and other cell structures might be better distinguished.

Conclusions

Until now, CLE has not been firmly established in pulmonology. Indeed, encouraging data in this field are increasing. In this study, we showed that BALF components (alveolar macrophages and leucocytes) are differentiable by CLE-based analysis, offering a new diagnostic approach.

References:

1. Kiesslich R, Goetz M, Lammersdorf K et al: Chromoscopy-guided endomicroscopy increases the diagnostic yield of intraepithelial neoplasia in ulcerative colitis. Gastroenterology, 2007; 132(3): 874–82

2. Filip MM, Gheonea DI, Georgescu CV et al: Quadri-modal imaging for real-time diagnosis of early gastric cancer. J Gastrointestin Liver Dis, 2009; 18(3): 271–72

3. Kiesslich R, Burg J, Vieth M et al: Confocal laser endoscopy for diagnosing intraepithelial neoplasias and colorectal cancer in vivo. Gastroenterology, 2004; 127(3): 706–13

4. Leggett CL, Gorospe EC: Application of confocal laser endomicroscopy in the diagnosis and management of Barrett's esophagus. Ann Gastroenterol, 2014; 27(3): 193–99

5. Tontini GE, Mudter J, Vieth M et al: Confocal laser endomicroscopy for the differential diagnosis of ulcerative colitis and Crohn's disease: A pilot study. Endoscopy, 2015; 47(5): 437–43

6. Thiberville L, Salaun M, Lachkar S et al: Confocal fluorescence endomicroscopy of the human airways. Proc Am Thorac Soc, 2009; 6(5): 444–49

7. Danilevskaya O, Averyanov A, Lesnyak V et al: Confocal laser endomicroscopy for diagnosis and monitoring of pulmonary alveolar proteinosis. J Bronchology Interv Pulmonol, 2015; 22(1): 33–40

8. Salaun M, Roussel F, Hauss PA et al: In vivo imaging of pulmonary alveolar proteinosis using confocal endomicroscopy. Eur Respir J, 2010; 36(2): 451–53

9. Shafiek H, Fiorentino F, Cosio BG et al: Usefulness of bronchoscopic probe-based confocal laser endomicroscopy in the diagnosis of Pneumocystis jirovecii pneumonia. Respiration, 2016; 92(1): 40–47

10. Salaun M, Roussel F, Bourg-Heckly G et al: In vivo probe-based confocal laser endomicroscopy in amiodarone-related pneumonia. Eur Respir J, 2013; 42(6): 1646–58

11. Yserbyt J, Dooms C, Decramer M, Verleden GM: Acute lung allograft rejection: Diagnostic role of probe-based confocal laser endomicroscopy of the respiratory tract. J Heart Lung Transplant, 2014; 33(5): 492–98

12. Fuchs FS, Zirlik S, Hildner K, Schubert J et al: Confocal laser endomicroscopy for diagnosing lung cancer in vivo. Eur Respir J, 2013; 41(6): 1401–8

13. Sorokina A, Danilevskaya O, Averyanov A et al: Comparative study of ex vivo probe-based confocal laser endomicroscopy and light microscopy in lung cancer diagnostics. Respirology, 2014; 19(6): 907–13

14. Hassan T, Piton N, Lachkar S et al: A novel method for in vivo imaging of solitary lung nodules using navigational bronchoscopy and confocal laser microendoscopy. Lung, 2015; 193(5): 773–78

15. Su Z, Zhong C, Li S et al: Needle-based confocal laser endomicroscopy in the diagnosis of peripheral pulmonary nodule: A preliminary report. J Thorac Dis, 2017; 9(8): 2608–12

16. Newton RC, Kemp SV, Yang GZ et al: Imaging parenchymal lung diseases with confocal endomicroscopy. Respir Med, 2012; 106(1): 127–37

17. Skold CM, Hed J, Eklund A: Smoking cessation rapidly reduces cell recovery in bronchoalveolar lavage fluid, while alveolar macrophage fluorescence remains high. Chest, 1992; 101(4): 989–95

18. Ghio AJ, Sangani RG, Brighton LE, Carson JL: MRT letter: Auto-fluorescence by human alveolar macrophages after in vitro exposure to air pollution particles. Microsc Res Tech, 2010; 73(6): 579–82

19. Thiberville L, Salaun M, Lachkar S et al: Human in vivo fluorescence microimaging of the alveolar ducts and sacs during bronchoscopy. Eur Respir J, 2009; 33(5): 974–85

20. Brownback KR, Thomas LA, Simpson SQ: Role of bronchoalveolar lavage in the diagnosis of pulmonary infiltrates in immunocompromised patients. Curr Opin Infect Dis, 2014; 27(4): 322–28

21. Gheonea DI, Cartana T, Ciurea T et al: Confocal laser endomicroscopy and immunoendoscopy for real-time assessment of vascularization in gastrointestinal malignancies. World J Gastroenterol, 2011; 17(1): 21–27

22. Sazonov DV, Ivanov IuV, Panchenkov DN et al: [Confocal laser endomicroscopy in the diagnosis of diseases of biliary ducts]. Eksp Klin Gastroenterol, 2014; (3): 25–31 [in Russian]

23. Pirogov SS, Sokolov VV, Karpova ES et al: [Early gastric cancer and precancerous conditions diagnostics with confocal laser endomicroscopy]. Eksp Klin Gastroenterol, 2014; (3): 18–24 [in Russian]

24. Bruno MJ: Novel diagnostic approach to pancreatic cysts: Is confocal laser endomicroscopy bridging the gap? Endoscopy, 2015; 47(1): 4–5

25. Caillol F, Bories E, Autret A et al: Evaluation of pCLE in the bile duct: final results of EMID study: pCLE: Impact in the management of bile duct strictures. Surg Endosc, 2015; 29(9): 2661–68

26. Gong S, Ge ZZ, Xue HB: In vivo diagnosis of gastric signet-ring cell carcinoma by confocal laser endomicroscopy. J Dig Dis, 2014; 15(1): 46–49

27. Nakai Y, Isayama H, Shinoura S et al: Confocal laser endomicroscopy in gastrointestinal and pancreatobiliary diseases. Dig Endosc, 2014; 26(Suppl. 1): 86–94

28. Gheonea DI, Saftoiu A, Ciurea T et al: Confocal laser endomicroscopy of the colon. J Gastrointestin Liver Dis, 2010; 19(2): 207–11

29. Li CQ, Yu T, Zuo XL et al: Effects on confocal laser endomicroscopy image quality by different acriflavine concentrations. J Interv Gastroenterol, 2011; 1(2): 59–63

30. Sanduleanu S, Driessen A, Gomez-Garcia E et al: In vivo diagnosis and classification of colorectal neoplasia by chromoendoscopy-guided confocal laser endomicroscopy. Clin Gastroenterol Hepatol, 2010; 8(4): 371–78

31. Fritscher-Ravens A, Schuppan D, Ellrichmann M et al: Confocal endomicroscopy shows food-associated changes in the intestinal mucosa of patients with irritable bowel syndrome. Gastroenterology, 2014; 147(5): 1012–20e4

32. Chen SP, Liao JC: Confocal laser endomicroscopy of bladder and upper tract urothelial carcinoma: A new era of optical diagnosis? Curr Urol Rep, 2014; 15(9): 437

33. Chang TC, Liu JJ, Liao JC: Probe-based confocal laser endomicroscopy of the urinary tract: the technique. J Vis Exp, 2013; (71): e4409

34. De Palma GD, Esposito D, Luglio G et al: Confocal laser endomicroscopy in breast surgery: A pilot study. BMC Cancer, 2015; 15: 252

35. Mooney MA, Zehri AH, Georges JF, Nakaji P: Laser scanning confocal endomicroscopy in the neurosurgical operating room: A review and discussion of future applications. Neurosurg Focus, 2014; 36(2): E9

36. Breuskin D, Divincenzo J, Kim YJ et al: Confocal laser endomicroscopy in neurosurgery: A new technique with much potential. Minim Invasive Surg, 2013; 2013: 851819

37. Just T, Pau HW: Intra-operative application of confocal endomicroscopy using a rigid endoscope. J Laryngol Otol, 2013; 127(6): 599–604

38. Pogorzelski B, Hanenkamp U, Goetz M et al: Systematic intraoperative application of confocal endomicroscopy for early detection and resection of squamous cell carcinoma of the head and neck: A preliminary report. Arch Otolaryngol Head Neck Surg, 2012; 138(4): 404–11

39. Thiberville L, Salaun M: Bronchoscopic advances: On the way to the cells. Respiration, 2010; 79(6): 441–49

40. Yserbyt J, Alame T, Dooms C, Ninane V: Pulmonary alveolar microlithiasis and probe-based confocal laser endomicroscopy. J Bronchology Interv Pulmonol, 2013; 20(2): 159–63

41. Ji R, Li YQ, Gu XM et al: Confocal laser endomicroscopy for diagnosis of Helicobacter pylori infection: A prospective study. J Gastroenterol Hepatol, 2010; 25(4): 700–5