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CRAIOVA**

DOCTORAL SCHOOL



DOCTORAL THESIS

- ABSTRACT -

**EVALUATION OF ANGIOGENESIS IN HUMAN
COLORECTAL CARCINOMA BY USING
EX VIVO - IN VIVO CONFOCAL LASER
ENDOMICROSCOPY (CLE) AND
IMMUNOHISTOCHEMISTRY**

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TABLE OF CONTENTS

STATE OF KNOWLEDGE

CHAPTER I

Epidemiology of colorectal cancer

CHAPTER II

Current innovations for the management of colorectal cancer: from prevention and diagnosis to treatment and monitoring

II.1. Colorectal cancer screening

II.2. Advanced endoscopic imaging techniques for improved detection of colorectal cancer

II.3. Clinical applications for early detection of colorectal neoplasia

II.4. Assessment of tumor margins and invasion depth

II.5. Current therapeutic options in colorectal cancer

CHAPTER III

Angiogenesis-based prognostic markers in immunohistochemistry for colorectal cancer patients

PERSONAL CONTRIBUTIONS

OBJECTIVES

MATERIALS AND METHODS. RESULTS. DISCUSSIONS

1. Immunohistochemical study - The role of the endothelial markers CD31 and CD105 in colorectal cancer

2. Endomicroscopic study (Confocal Laser Endomicroscopy)

2.1. Evaluation of new morphometric parameters of neoangiogenesis in human colorectal cancer using confocal laser endomicroscopy (CLE) and targeted panendothelial markers

2.2. Neoangiogenesis evaluation of colorectal cancer using confocal laser endomicroscopy and anti-CD105 antibodies

2.3. Endomicroscopy with fluorescent CD105 antibodies for in vivo imaging of colorectal cancer angiogenesis

FINAL CONCLUSIONS

REFERENCES

KEYWORDS

Colorectal cancer, neoangiogenesis, morphometric analysis, panendothelial markers, anti-CD105 antibodies, advanced endoscopic imaging techniques, confocal laser endomicroscopy.

STATE OF KNOWLEDGE

CHAPTER I

Epidemiology of colorectal cancer

Colorectal cancer has the highest chance of curability from all gut malignancies as long as it is detected at an early stage, before lymph node involvement. It remains the second cause of death worldwide from all cancers, although it is one of the malignant diseases with a proven benefit from targeted therapy [1, 2]. Despite a large number of already existing studies dealing with either antibodies or gene expression profiling as potential prognostic biomarkers and with various clinical trials of innovative targeted therapies, increased incidence and mortality of the disease are still a challenge.

CHAPTER II

Current innovations for the management of colorectal cancer: from prevention and diagnosis to treatment and monitoring

Early detection together with an accurate staging of cancer is the cornerstone that leads to a decrease in costs and invasiveness of the curative techniques and further to significantly improvement of patients' outcome. *Conventional white-light endoscopy*, currently regarded as a gold standard for colorectal cancer detection and as a preoperative marking tool, is far from being an ideal tool to detect, characterize, and confirm the nature of a colorectal lesion at the bedside in order to indicate targeted biopsies or resections only when necessary. Therefore, a complex approach that includes efficient screening programmes with modern minimally invasive and cost efficient diagnostic and therapeutic procedures is necessary.

The main purpose of this chapter is to address minimally invasive endoscopic imaging techniques for preventing, diagnosis, staging and monitoring of colorectal cancer, with emphasis on a novel promising clinical tool, *confocal laser endomicroscopy*.

Colorectal cancer screening

Implementation of screening programs such as fecal occult blood test, colonoscopy or sigmoidoscopy improved the chances of detection and removal of adenomatous polyps as well as of early diagnosis of cancer with a better outcome for the patient.

Prevention is based on diagnosis of either potentially curable colorectal neoplasia in an early stage or of precursor conditions with significant risk of progression to cancer. Easy and accurate detection of dysplasia, early detection of malignancies and accurate discrimination of non-neoplastic lesions or of inflammatory disease from neoplasia remain the focus in endoscopy [3]. Various innovative imaging techniques using endoscopy have been developed in order to detect lesions that are still invisible under conventional methods. Beyond all, currently, histology is still required to clarify the diagnosis [3].

Confocal laser endomicroscopy (CLE) has rapidly gained momentum, being taken into consideration as a potential enhancement feature of conventional endoscopy which offers the possibility of *in vivo* optic biopsies, facilitating real-time histological examination of the superficial layer of the gastrointestinal tract. Extended studies are underway to determine the efficiency of this technique in the screening, follow-up and early diagnosis of colon lesions. Presently, the use of confocal laser endomicroscopy in screening and long-term surveillance is limited by the narrow field of view, the reduced tissue penetration, as well as the prohibitive financial costs [4, 5].

Advanced endoscopic imaging techniques for improved detection of colorectal cancer

Confocal laser endomicroscopy (CLE) is a promising technology for *in vivo* early detection of gastrointestinal mucosal changes. A system based on a laser microscope with dedicated confocal miniprobes (pCLE, Cellvizio, Mauna Kea Technologies, Paris, France) inserted through the accessory channel of a traditional endoscope is compatible with all flexible video-endoscopes and enables a seamless procedure session from diagnosis and therapeutic such as EMR [6]. Ultra high definition (UHD) ColoFlex™ is the latest model of pCLE probes dedicated for colonoscopy. Compared with other new optic techniques, the greatest advantage of CLE is that it can enable surface and subsurface imaging of living cells in the mucosa during ongoing endoscopy.

Clinical applications for early detection of colorectal neoplasia

Studies based on **CLE** as a promising clinical tool to detect neoplasia in the gastrointestinal tract are currently increasingly reported [6]. pCLE- based Miami classification system is now considered to be the standard reference which describes pCLE criteria for normal mucosa, hyperplastic polyps, adenomas and adenocarcinomas [7]. Moreover, **fluorescein- guided endomicroscopy** can be used to differentiate non-neoplastic from neoplastic lesions through the *morphometric pattern of the microvascular network*.

Recently, in a preliminary consensus report based on clinical evidence regarding the role of CLE in colorectal cancer experts agreed that CLE is highly accurate for real-time histopathological classification of colonic neoplasia *in situ* and that CLE criteria can be used to accurately detect from normal to hyperplastic/adenomatous (dysplastic) polyps and to cancerous mucosa; excepting criteria for serrated neoplasia where further validation is needed [8].

With the advancements made in colorectal cancer, the need for additional markers that improve the prognostic and predictive power of the pathologic analysis of the primary lesion is of major interest. Therefore, another promising alternative for early colorectal cancer detection would be the use of **peptids conjugated with fluorescent agents** that target the dysplastic cells as an adjunct to CLE. *We have demonstrated the feasibility of CLE for imaging different aspects of the tumor vasculature from fresh biopsy samples similar to the currently accepted histopathology techniques by using CLE combined with fluorescently labeled panendothelial markers (CD31) and selective markers (CD105) for labeling of both*

normal and tumor blood vessels in patients with colorectal cancer [9, 10]. The feasibility of the method on human subjects was also tested in vivo [11]. This could add functional analysis to the morphological aspect of the neoplastic process and could serve for possible applications from diagnosis to prognosis stratification and targeted treatment.

Assessment of tumor margins and invasion depth

It was also stated that **CLE** can be used to classify lesions and define margins for EMR/ESD, while real-time **CLE combined with virtual chromography** could guide the extent of therapy for residual neoplasia post-EMR [12]. Moreover, CLE alone was found sufficient to guide therapeutic resection strategy in case of diagnosis of intramucosal carcinoma or high-grade dysplasia [69]. Lately, a consensus of experts stated that pCLE should be considered in the diagnosis of intramucosal neoplasia to trigger a therapeutic decision, the evaluation of the extent of the lesion and the associated therapy as well as for post-resection surveillance, but needs to be recognized in the guidelines of scientific societies in order to be applied in daily clinical practice [13].

Current therapeutic options in colorectal cancer

In advanced stages this malignancy has a poor prognosis due to the inefficacy of the existent treatment and at this point the outcome cannot be completely predicted by common prognostic factors such as lymph node involvement, tumor size or local extension of the disease. As angiogenesis plays a key role in the development and dissemination of colorectal cancer, *antiangiogenic inhibitors* represent a promising strategy for the management of these patients.

CHAPTER III

Angiogenesis-based prognostic markers in immunohistochemistry for colorectal cancer patients

One increasingly frequent purpose of IHC, beyond the confirmation of tumor diagnosis, has become to provide useful prognostic information and even predict responsiveness to standard chemotherapy or novel molecular targeted therapy [14].

To date, a number of publications on immunohistochemical analysis of blood vessels in colorectal cancer and the prognostic value of different *angiogenesis-based parameters* have shown controversial results. Apparently, the main cause of this aspect could be due to variations in the methods of analysis and the choice of various markers. Variations in the mechanisms of proliferation of blood vessels in tumors and in the spectrum of immunoreactivity of each marker and duration of the follow-up period could also be implied [15].

With further progression towards antibody-based therapeutic strategies in cancers, several potential targets have already been described but only a few have provided prognostic data [16]. Thus, to improve patient outcome and understanding of the natural course of the disease, it is mandatory to further define prognostic markers which may, in addition, also serve as therapeutic targets.

Prognostic biomarkers currently used in immunohistochemistry, are expected to be translated into imaging biomarkers in colorectal cancer by using techniques such as confocal laser endomicroscopy. Obviously, there is still room left for improvement in the “*in vivo*” application of these methods to validated biomarkers [9].

The aim of this chapter is to address current immunohistochemical angiogenesis-based prognostic markers for patients with colorectal cancer, to summarize their current state and to discuss future perspectives [17].

PERSONAL CONTRIBUTIONS

The prospective study included a total of 59 patients with colorectal tumors diagnosed in the Gastroenterology and Hepatology Research Center, as well as in the 1st and 3rd Surgery Clinics of The Emergency Clinical County Hospital of Craiova, between December 2011 and March 2015.

1. Immunohistochemical study - The role of the endothelial markers CD31 and CD105 in colorectal cancer

The purpose of this study was to assess the accuracy of two angiogenesis markers, CD31 and CD105, as potential prognostic indicators in colorectal cancer by evaluating their association with clinical and pathological factors.

Materials and Methods: The immunohistochemistry study was realized on 43 patients from the entire group (37 men, 6 women), diagnosed with colorectal cancer, who underwent curative surgery. The specimens were fixed in 4% neutral buffered formalin and processed for routine paraffin embedding and sectioning as 4 µm-thick sections. From each block, slides were stained with hematoxylin-eosin for both pathological diagnosis and for immunohistochemistry to visualize the CD31 and CD105 antigens. Thus, vascular area and vessel density (MVD) were determined on a total of 380 images and the results were normalized for 1 mm² areas and expressed as the mean ± standard error. For MVD values and vascular surface, we initially recorded the average ± standard deviation, normalized at 1mm², and, consequently, we evaluated the relationship between these two vascular parameters and clinicopathological factors (survivability, presence of anemia and signs of inflammation such as leukocytosis/increased erythrocyte sedimentation rate at the moment of diagnosis, postoperative tumor stage – pTNM, tumor grading – G) by using the Student t-test and ANOVA.

Results: Regarding MDV – CD105, we identified a statistically significant difference between deceased patients and survivors ($p=0.008$). On the other hand, in the case of MDV – CD31, the results did not support the existence of a significant difference between the two groups ($p = 0.060$). Furthermore, our analysis revealed that the percentage of vascularized area for CD 31 positive patients that presented signs of inflammation at the time of diagnosis was statistically different compared to that of patients that did not presented an inflammatory syndrome ($p=0.003$).

Conclusions: The panendothelial marker CD31, frequently used for quantifying MVD, is not an accurate indicator of tumor angiogenesis. Conversely, the assessment of tumor angiogenesis using CD105 may be used as an independent biomarker for determining patient prognosis and individualized adjuvant therapy in colorectal cancer as we have identified a correlation between MVD-CD105 and patient mortality. The immunohistochemical study is limited by the fact that the activity of markers with specificity for the activated endothelium, such as CD105, are dependent on the quality of the fixation process for biopsies included in paraffin.

2. Endomicroscopic study (CLE)

2.1. Evaluation of new morphometric parameters of neoangiogenesis in human colorectal cancer using confocal laser endomicroscopy (CLE) and targeted panendothelial markers

The aim of this study was to evaluate new vascular morphometric parameters in colorectal cancer, difficult to achieve through conventional immunohistochemistry, by using the confocal laser endomicroscopy method.

Materials and Methods: In this prospective study, from a total of 59 patients included with colorectal cancer, 17 patients (13 men and 4 women) with a mean of age of 61 years \pm 12.8, accepted to sign the informed consent for the CLE evaluation of the fresh biopsies collected from their colon. Initially, the study group comprised 13 patients with rectal adenocarcinomas, one patient with history of malignant neoplasm of rectosigmoid junction four years before and diagnosed with local recurrence at follow-up, one patient with sigmoid colon cancer, one with cancer of the descending colon and one patient with cancer of the ileocecal valve. Finally, 3 of the patients who presented distant metastases (2 patients with liver metastases and one with pulmonary metastases) were excluded from the study.

Thus, fresh biopsies from tumor and normal tissue were collected during colonoscopy from 14 patients with T3 staged, well to moderately differentiated carcinoma (G1–G2 grade), without metastasis (13 patients with rectal adenocarcinomas and one with cancer of the rectosigmoid junction) and were marked with fluorescently labeled anti-CD31 antibodies. A series of optical slices spanning 250 μ m inside the

tissue were immediately collected for each sample using a confocal laser endomicroscope. All measurements were expressed as the mean \pm standard error.

Results: The mean diameter of tumor vessels was significantly larger than the normal vessels ($10.41 \pm 0.5 \mu\text{m}$ vs. $7.65 \pm 0.4 \mu\text{m}$, $p=0.0005$). The vessel density was also significantly higher in the cancer vs. normal tissue samples (5022.5 ± 344 vs. 3452.6 ± 279.7 vessels/ mm^3 , $p=0.0015$). These results were confirmed by immunohistochemistry. In addition, the tortuosity index and vessel lengths were not significantly different (1.04 ± 0.03 and $30.88 \pm 1.97 \mu\text{m}$ in normal tissue, vs. 0.9 ± 0.02 and $26.08 \pm 1.5 \mu\text{m}$ in tumor tissue respectively, $p=0.608$ and $p=0.949$). The daughter/mother ratio (ratio of the sum of the squares of daughter vessel radii over the square of the mother vessel radius) was 1.22 ± 0.06 in normal tissue, and 1.17 ± 0.05 in tumor tissue ($p=0.932$).

Discussions and conclusion: Typically, in tumor vascular studies the microvasculature is described only from the vessel diameter and density points of view because these features can be measured from histological slices. We have also performed an immunohistochemical study on our samples, and obtained similar results to the literature (larger MVD and vascular area in tumors vs. normal tissue) [18]. In addition, we evaluated more parameters of vascularization from volumetric samples, such as the vessel length, the index of tortuosity and the daughter/mother ratio, that can be easily quantified with CLE due to its greater penetration depth into the tissue (up to $250 \mu\text{m}$). *To our knowledge, this is the first report of the daughter/mother ratios and tortuosity index in colorectal cancer vasculature.* In our confocal optical sections, the malignant blood vessels appeared more dilated and more tortuous compared to the normal samples. Interestingly, in spite of the visual examination, we obtained no statistical significance when we calculated their index of tortuosity. This could reflect the fact that the tortuous appearance might be given by the mechanical deformation as the entire tumor tissue grows rather individual vessel fragments being tortuous.

One limitation of our study is that the direction of blood flow is not known, therefore the exact designation of the mother vessel cannot be specified. This shortcoming could be addressed with the *in vivo* observation of blood flow in tumors, using clinically approved contrast agents. Other methodology could be applied to studies which monitor the effect of anti-angiogenesis therapy (e.g. bevacizumab) on vascular branching, tortuosity and vessel volume in tumors.

Taking into consideration the fact that one strategy for successful treatment with anti-angiogenic agents is to reduce the tortuosity of the abnormal vessels [19] and that the efficacy of chemotherapy depends on the presence of an adequate tumor blood supply to ensure drug delivery, “normalization” of the tumor vascular network is desirable. Thus, assessing the differences between the vessel length, tortuosity and branching patterns with CLE imaging and fresh biopsy staining could be useful for identifying the category of patients who might benefit from neoadjuvant angiogenetic therapy [20].

Therefore, confocal laser endomicroscopy is feasible for measuring more vascular parameters from fresh tumor biopsies than conventional immunohistochemistry alone. Provided new contrast agents will be clinically available, future *in vivo* use of CLE could lead to identification of novel biomarkers based on the morphometric characteristics of tumor vasculature.

2.2. Neoangiogenesis evaluation of colorectal cancer using confocal laser endomicroscopy and anti-CD105 antibodies

Aim: The aim of this study was to evaluate neoangiogenesis in patients with colorectal cancer by two fluorescently labeled antibodies on fresh biopsy samples imaged with CLE. More specifically, we aimed to answer the following questions: 1) Can the use of CLE in association with CD105 offer a more adequate quantitative and qualitative analysis of newly formed vessels than the commonly used pan-endothelial markers in human rectal cancer? and 2) Can this method be used *in vivo* for a rapid characterization of tumor microvascularization?

Materials and Methods: An important question in validating tumor angiogenesis is what proportion of the tumor vascular network is represented by pre-existing parent tissue vessels and newly formed vessels. CD105 (endoglin) represents a proliferation-associated endothelial cell adhesion molecule. In contrast to pan-endothelial markers, such as CD31, CD105 is preferentially expressed in activated endothelial cells that participate in neovascularization. Thus, we evaluated CD105 and CD31 expression from samples of patients with primary rectal adenocarcinoma, using a dedicated endomicroscopy system. Tissue specimens from ten patients 47–80 years old (mean age of 65.2 ± 9.9 years), with histologically diagnosed rectal cancer, were collected during colonoscopy before undergoing surgical resection or neoadjuvant therapy to avoid artifacts (e.g. false positive resulted from fibrosis or inflammation increased in case of radio-chemotherapy). Fresh tissue samples from these patients were immediately processed for both CLE and immunohistochemistry assessment. The ten patient population contained stage II-III (according to AJCC staging system) rectal adenocarcinomas without metastatic spread.

An imaging software was used to obtain the Z projection of the confocal serial images from each biopsy sample previously combined into stacks. Vascular density and vessel diameters were measured within two $50 \times 475 \mu\text{m}$ rectangular regions of interest centered in the middle of each image in the horizontal and vertical direction. The results were averaged over all the patients and were expressed as the mean \pm standard error.

Results: The use of an anti-CD105 antibody was found to be suitable for the detection of blood vessels in colon cancer. Whereas anti-CD31 antibodies stained blood vessels in both normal and pathologic colon equally, CD105 expression was observed primarily in malignant lesions, with little or no expression in the vessels of the normal mucosa (244.21 ± 130.7 vessels/ mm^3 in only four patients). The average diameter of anti-CD105 stained vessels was $10.97 \pm 0.6 \mu\text{m}$ in tumor tissue, and the vessel density was 2787.40 ± 134.8

vessels/mm³. When using the anti-CD31 antibody, the average diameter of vessels in the normal colon tissue was 7.67±0.5 µm and the vessel density was 3191.60±387.8 vessels/mm³, while in the tumors we obtained an average diameter of 10.88±0.8 µm and a vessel density of 4707.30±448.85 vessels/mm³. Thus, there were more vessels stained with CD31 than CD105 (p<0.05). The average vessel diameter was similar for both CD31 and CD105 staining. A qualitative comparison between CLE *versus* immunohistochemistry lead to similar results.

Conclusions: Specific imaging and quantification of tumor microvessels are feasible in human rectal cancer using CLE examination and CD105 immunostaining of fresh tissue samples. *To our knowledge, this is the first study to report the use of fluorescently-labeled CD105 antibody in conjunction with CLE in patients with rectal tumor.*

A larger number of patients is needed to study the correlation between MVD and tumor differentiation grade and stage, with great potential for CD105 staining combined with CLE analysis to provide a more reliable evaluation of the angiogenetic status of patients with colorectal cancer.

2.3. Endomicroscopy with fluorescent CD105 antibodies for in vivo imaging of colorectal cancer angiogenesis

Aims: We had previously proposed CD105 in conjunction with CLE as a more reliable tool for real-time evaluation of the angiogenetic status of patients with colorectal cancer, demonstrating that specific imaging of tumor microvessels is feasible using *ex vivo* CLE examination and CD105 immunostaining on fresh tissue samples [10]. This study illustrates the feasibility of *in vivo* application of fluorescent antibodies for molecular imaging on human patients with colorectal cancer, before further adaptation of the method to targeted therapy.

Materials and Methods: The patient (65 years old) was recruited from Department of Surgery, Emergency County Hospital of Craiova, immediately before undergoing surgical intervention for rectal cancer. The study was conducted according to the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Tumor was washed with saline solution and it was evaluated from a stable position using an eCLE system, before undergoing surgery. After excluding the presence of tissue autofluorescence, 1 ml fluorescent antibody solution (FITC-labeled anti-CD105/Endoglin antibody, Exbio, 1:5) was topically administered through a spray-catheter. After 10 min of incubation, the targeted area was analyzed by CLE and images were recorded. The fractal dimension of tumor vessels was calculated offline using “fractal box count” tool in Image J software on the confocal serial images converted to RGB stacks. Vascular density, generally evaluated in immunochemistry by estimating the image area covered by vessels, was also estimated, as a nonfractal parameter. Before *in vivo* testing, multiple attempts were made at establishing the optimal antibody-tissue contact time using paraffin-embedded tissue sections under

fluorescence microscopy conditions. Corresponding tumor biopsy samples underwent immunohistochemical staining for CD105 and CD31, assessing microvessel density (MVD) and vascular areas. Fractal analysis of vascular network was assessed on binarized - skeletonized masks by using the fractal box counting method in ImageJ.

Results: In vivo CLE analysis of CD105 expression in human colorectal cancer enabled the study of vascular network, revealing a chaotic structure. Both good and medium quality images were eligible for clinical analysis (n=67 images). Fractal value was 1.46, indicating the chaotic architecture (with 2 being the fractal value for normal vessels). Tumor vessel density was 146.09 vessels/mm². No immunological side effects or other adverse events occurred when anti-CD105 antibodies conjugated with FITC have been administered topically *in vivo*. Immunohistochemistry evaluation confirmed the presence of blood vessels stained with commonly used CD31 as well as with CD105, with less vessels detected by using CD105 than by using CD31 staining. CD31 allowed a count of 294.3 tumor vessels/mm² and a vascular area of 21.4%. There was a count of 239.55 tumor vessels/mm² due to CD105 expression with a vascular area of 5.28%. Fractal dimension was 1.12 for CD105 stained images. Standard histological assessment showed a moderately differentiated adenocarcinoma (G2) with mucinous areas. Postoperative stage of the tumor was T3N1M0.

Conclusion: In vivo molecular imaging of human colorectal cancer neoangiogenesis using CLE and FITC-CD105 antibodies is feasible. *To the best of our knowledge, this is the first report of in vivo CLE imaging of vascularization in human colorectal cancer using CD105.*

Image accuracy could have been influenced by several factors. In our study, the time interval from the topic administration of antibodies to CLE examination was of 10 minutes. Given the fact that valid time interval of ideal antibody binding is dependent on the molecular target and the route of administration [21], further studies in vivo with various duration attempts are necessary for the standardization of the protocol. Other possible factors influencing image quality include CLE probe not placed properly, inadequate quantity of FITC-CD105, overexposure to laser power (e.g. the laser power was not turn off in time when it was not in use) the increased heat production of the laser light, embarrassing continuous observation of the same area for longer periods of time by phototoxic effect induced in the tissue. Therefore, by controlling these situations, CLE image quality could be improved so as to facilitate better *in vivo* detection of pathological changes.

Future *in vivo* applications of immunoendoscopy using CD105 could provide a more specifically analysis of tumor microvascular architecture in order to improve diagnosis, patient stratification and monitoring of antiangiogenic therapy.

FINAL CONCLUSIONS

- The histopathological study revealed an increased incidence of colorectal adenocarcinoma in the 50 years age group, as well as a clear predominance of this neoplasia in the male population. The majority of cases presented rectal tumors with a postoperative T3 tumor stage, with neither lymph node metastases, nor distant metastases. Most of the adenocarcinomas were moderately differentiated (G2).
- The panendothelial marker CD31, frequently used for quantifying MVD, is not an accurate indicator of tumor angiogenesis. In the case of MDV – CD31, the results did not support the existence of a significant difference between deceased patients and survivors at the end of the study. Furthermore, our analysis revealed that the percentage of vascularized area for CD 31 positive patients that presented signs of inflammation at the time of diagnosis was statistically different compared to that of patients that did not presented an inflammatory syndrome.
- The assessment of tumor angiogenesis using CD105 may be used as an independent biomarker for determining patient prognosis and individualized adjuvant therapy in colorectal cancer as we have identified a correlation between MVD-CD105 and patient mortality. These results require further research, especially through prospective studies on homogenous groups of patients.
- Confocal laser endomicroscopy associated with panendothelial fluorescent markers applied on fresh unfixed tissue biopsies allows the measurement of more vascular parameters than standard immunohistochemistry.
- The major advantages of this method are represented by the faster processing time and the absence of artefacts that may be generated by the fixation techniques used in immunohistochemistry. A 2D section does not offer the possibility of an accurate measurement of blood vessel length, tortuosity index or daughter blood vessel/mother blood vessel ratio.
- This endoscopic imaging technique (CLE) combined with targeted antibodies can be used for evaluating tumor 3D microvascular architecture, being able to quantitatively and qualitatively analyze tissue angiogenesis. However, this method requires further *in vivo* studies focused on the morphological tumor development and its relation to the micro-vascularization variations in time.
- Specific detection and quantification of tumor micro-vascularization is feasible *ex vivo* and *in vivo* with the help of CLE associated with CD105 immunostaining of colorectal tissue.

- Targeted angiogenesis markers in combination with CLE examination may present a viable option for the evaluation of the angiogenic status in patients with colorectal cancer.

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