

**UNIVERSITY OF MEDICINE AND PHARMACY CRAIOVA  
DOCTORAL SCHOOL**

**PhD THESIS  
ABSTRACT**

**CLINICAL, HISTOLOGICAL AND  
IMMUNOHISTOCHEMICAL STUDY OF  
COLORECTAL CARCINOMAS**

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# **INTRODUCTION**

Colorectal cancer (CRC) represents the second most common cause of cancer in women and third most common cause in men in most developed countries. Also, it accounts for most of the cancer-related deaths after lung, liver, and stomach cancer. Even though the CRC incidence is stabilizing or declining in previous high-risk countries, there appears to be an ascending trend of CRC incidence in low-risk areas, due to several factors, which include diet changes, smoking and obesity. The CRC mortality rates are evidently decreasing worldwide, which, in most part, is attributed to increased efficiency of the screening methods, improved treatment and reduced prevalence of risk factors. However, because of the limited healthcare resources combined with an increase in CRC incidence, in Romania mortality rates of colorectal cancer are still increasing.

The tumor stage (TNM), the histological type / subtype, the degree of differentiation and the interest of the lymph nodes, the vascular and perineural invasion represent the most important histopathological parameters, as the immunohistochemical data underline the prognostic role of angiogenesis in colorectal cancer, the relationship between angiogenesis and survival. Both increased microvascular density and vascular endothelial growth factor expression have been shown to be independent prognostic factors independent of tumor stage, correlating with lower overall survival, but especially with a shorter progression free interval.

## **CHAPTER 1**

### **Large bowel anatomy and histophysiology**

#### **1.1. Macroscopic aspects of the colon and rectum**

The large intestine is the last portion of the digestive tract, continuing the small intestine and opening outward through the anus. It is located in the peritoneal cavity, between the ileocecal valve and the anus, with a length of about 1.60 m, a diameter that decreases from 7 cm (originally) to 3-3.5 cm (the terminal portion) and surrounds the small bowel as a frame. The structure of the large intestine is adapted to the storage function of the residues resulting from the digestion process.

The segmentation of the large intestine is made according to several criteria: ontogenetic development, physiological aspects, particularities determined by surgical practice. The anatomico-surgical criterion is very important, being dependent on vascular territory.

## **1.2. General organization of subdiaphragmatic digestive tube**

From the esophagus to the anal orifice, the digestive tract presents a tubular disposition whose diameter differs according to the anatomical segment studied. Its components are divided into an supradiaphragmatic region (the pharyngeal-esophageal duct) that continues the oral cavity and a subdiaphragmatic (gastrointestinal tract) region. Apart from regional structural differences due to their functionality, the wall of the digestive tract presents a common structural organization, represented by four tunics: mucosa, submucosa, muscular and serosa.

## **1.3. Large bowel histology**

The large or terminal intestine (between 1.40 and 2m in length) is topographically divided into the cecum, appendix, colon, rectum, and anal canal. The structure of the large intestine wall has some changes to the rest of the segments, related to its function. Temporary storage of residual digestion residues occurs at this level; here are also processes of absorption, especially the absorption of water but also of small amounts of nutritional principles. Although the large intestinal mucosa does not produce enzymes, the digestion process continues at this level. Digestion in the intestinal tract is accomplished on the one hand by enzymes from the small intestine (which remain active) and, on the other hand, by the microbial flora of fermentation and putrefaction. Fermentation processes, through the bacterial aerobic flora found in the cecum, ascending colon, and the first half of the transverse colon, carry out cellulose digestion and the rotting through the anaerobic flora found in the rest of the colon, causes degradation of undigested proteins. Under the action of aerobic flora occurs in the large intestine synthesis of vitamins from complex B and vitamin K; degradation of undigested proteins under the action of anaerobic flora produces toxic substances for the organism. Most bacteria in the large intestine are anaerobic.

## **1.4. Large bowel vascularization and innervation**

The artery of the cecum and appendix originates in the ileocolic artery, which originates from the superior mesenteric artery. The artery of the cecum comes from the upper mesenteric artery, the ileocolic artery, the right colic artery, the middle colic artery and the lower mesenteric artery through the left colic artery and the sigmoid arteries. In the vicinity of the colon, the colic arteries anastomose and form the paracolic arcade, from which the right (long and short) arteries that irrigate the triangular areas of the colon, on the anterior and posterior sides of the colon.

Colic veins are born in the mucosa and muscular level, from where the veins that accompany the homonymous arteries flow into the upper and lower mesenteric veins, which flow into the portal vein.

The rectal arteries are rectal arteries, commonly called hemorrhoids. These are three, the upper, middle and lower rectal arteries. The most important of these is the upper rectal artery, the other being an accessory.

### **1.5. Large bowel histophysiology**

Although the main function is motricity, the large intestine also has digestive functions (absorption, secretion, food degradation), participating in the body's defense through the lymphatic follicles: motricity, absorption, secretion and digestion function.

## **CHAPTER 2**

# **COLORECTAL CANCER**

### **2.1. Colorectal cancer**

Colorectal cancer is an important public health issue because there are almost one million new cases of colorectal cancer diagnosed worldwide every year and half a million deaths caused by this condition. Recent reports show that in the United States, it was the most common form of cancer among people aged 75 years and over. Given that most cancers occur in older people, this observation gives additional impetus to investigating prevention and treatment strategies in this population subgroup. Although there are many questions to be solved, many facets of colorectal cancer are becoming increasingly understood and preventive perspectives are becoming apparent (World Health Organization, 2011).

Colorectal cancer is the third most common cancer in men (746,000 cases, 10.0% of the total) and second in women (614,000 cases, 9.2% of total) worldwide. Almost 55% of cases occur in more developed regions. There is a 10-fold variation in incidence across the world, and the patterns are very similar in males and females, with the highest rates in Australia / New Zealand (ASR 44.8 and 32.2 per 100,000 men and women respectively ), And the smallest in West Africa (4.5 and 3.8 per 100,000) (Bray F, 2014).

### **2.2. Colorectal cancer etiopathogenesis**

Colorectal cancer (CCR) develops through a multistage process, resulting from the progressive accumulation of genetic mutations, and frequently as a result of mutations in the Wnt signaling pathway. However, it has become evident over the last two decades that the epigenetic changes of the chromatine,

especially chromatin components in tumor suppressor and oncogene gene promoters, play a key role in the pathogenesis.

### **2.3. Mortality in colorectal cancer**

Colorectal cancer death rates have been decreasing since 1980 in men and since 1947 in woman.

### **2.4. Colorectal cancer staging**

The current staging system for colorectal cancer that should be used is the TNM classification system. The TNM system classifies colorectal tumors based on invasiveness (not the size) of the primary tumor (stage T), the number (not the size or location) of the loco-regional positive lymph nodes (stage N), and the presence or absence of metastatic disease (DeVita VT, Lawrence JTS, Rosenberg SA, 2014).

### **2.5. Colorectal cancer diagnosis**

Symptoms associated with colorectal cancer include gastrointestinal bleeding, changes in intestinal transit, abdominal pain, weight loss, appetite changes, and weakness, and in particular obstructive symptoms are alarming (Stein W, Farina A, Gaffney K et al. 1993). However, in addition to obstructive symptoms, other symptoms do not necessarily correlate with the disease state or predict a particular diagnosis (Majumdar SR, Fletcher RH, Evans AT, 1999).

After establishing the diagnosis and stage of the disease for both colon tumors and rectal tumors, it is essential to discuss the best therapeutic option with the medical oncologist, radiotherapist and surgical oncologist in order to formulate and implement an optimal treatment plan (DeVita VT, Lawrence JTS, Rosenberg SA, 2014).

### **2.6. Prognostic factors**

The Pathologists American College (PAC) has published a statement of consensus presenting their interpretation of the validity and usefulness of a large number of prognostic factors and prediction in colorectal cancer. Variables were categorized as belonging to categories I-IV (Compton CC, Fielding LP, Burgart LJ et al., 1999).

The most important prognostic factor remains today the TNM staging, also included in the CAP consensus declaration as a category I factor.

Although contradictory reports have been reported in the literature, the CAP consensus declaration has given vascular and lymphatic invasion as Category I, indicating that the predominance of the evidence strongly supports that their presence is unfavorable prognostic indicators.

## **CHAPTER III**

### **Clinico-epidemiologic and histopathological study of colorectal carcinoma**

#### **3.1. Materials and methods**

The study was analytical and prospective and included a total of 259 patients diagnosed with colorectal carcinoma (CRC) hospitalized in the surgical clinics of Craiova County Emergency Clinical Hospital and ONCOLAB Craiova Oncology Center for Investigation and Treatment in a two-year period, 2014-2015. The fragments processed for the histopathological study were obtained either from surgical resection or colonoscopy.

The criteria for inclusion in the study were the diagnosis of colorectal carcinoma, established by a histopathological examination performed in the pathology laboratory of the Craiova Emergency County Clinical Hospital and primitive tumor status, patients without history of chemotherapy, radiotherapy or immunotherapy.

#### **3.2. Results**

The median age for the men studied was 66.16 years, respectively 64.43 years for women, with average values being quite close (t-test: Two Sample Assuming Unequal Variances:  $p > 0.05$ , no statistical significance), but we can note Lower age for diagnosis in women than in males.

For the studied group we can state that there is no significant difference between the two sexes in terms of incidence according to the decades of age of the investigated patients.

Women showed the location of neoplasia in the right colon (cecum, ascending, hepatic flexion) in 37.64% of the cases studied, more frequently than in males, which had only 22.66% of the cases. In the transverse colon the percentage was higher for men 6% vs. 3.52% for women. In the left colon the

percentages were higher in males - 39.99% vs. 28.21% for women; It is also important to specify that the descending colon was the most obvious difference between the two sexes: males 28 cases - 18.66% while there were only 3 cases in women - 3.52%. Relatively equal proportions were found in the rectum and rectosigmoid junction: 30.58% for females and 31.32% for males.

The macroscopic aspect of the tumor was described in a total of 193 patients (74.23%), distributed as follows: vegetative tumors were highlighted in 23 cases - 11.91%, ulcero-vegetative tumors in 55 cases - 28.49%, ulcerative tumors 42 cases - 21.76%, infiltrating tumors 28 cases - 14.50%, ulcerated infiltrative tumors 39 cases - 20.20%, vegetative and infiltrative tumors 6 cases - 3.10%. The ulcerative component most frequently encountered, 136 cases - (70.46%).

Of the 178 patients to whom I was able to determine tumor size, 72 were women and 106 men. For tumors  $\leq 5$  cm for men, 70 cases (66.03%) were identified and for women 42 cases (58.33%); For tumors of 5-10 cm: 28 cases in males (26.41%), 20 cases in females (27.77%); Tumors  $\geq 10$ cm: 8 cases in males (7.54%) and 10 cases in females (13.88%). The overall mean tumor size in the patients studied was 5.26 cm, being slightly higher for women, 5.56 cm and 5.06 cm for men.

The histopathological type was established in 251 cases. The most common type of histopathology was adenocarcinoma without further specification, which was present in 225 patients (89.64%). In 14 cases histopathological diagnosis was mucinous adenocarcinoma (5.57%), in 5 cases (1.99%) the established histopathological diagnosis was tubulo-papillary adenocarcinoma, 2 cases of adenocarcinoma with vilous pattern (0.79%), 2 cases of small cell adenocarcinoma (0.79%), 1 case of squamous cell adenocarcinoma (0.39%), undifferentiated carcinoma (0.39%) and adenosquamos carcinoma (0.39%).

Of the 259 cases studied, 245 were eligible for tumor grading: 52 samples were classified as G1 (well differentiated) - 21.22%, 143 cases G2 (moderately differentiated) -58.37%, 49 cases G3 (poorly differentiated) -20% and one case G4 (undifferentiated) - 0.40%.

Globally, for both sexes, 253 patients were assessed for tumor stage: 2 patients staged T1 (0.79%), 37 with T2 (14.62%), 179 with T3 (70.75%), 33 patients with T4 tumor (13.04%). For men: 2 patients had classified tumors T1 (1.26%), 22 T2 tumor patients (13.92%), 111 T3 tumors (70.25%), 23 T4 tumor cases (14.55%). In women: none of the patients had a T1 class tumor, 15 patients had T2 (16.12%), 68 T3 (73.11%), 10 T5 (10.75%).

Examination of loco-regional lymph nodes was performed in 209 cases (80.69%). Their classification was based on the staging criteria developed by the American Joint Committee on Cancer as follows:

- N0 (no metastases in loco-regional lymph nodes): 79 cases - 30.50%;
- N1 (metastasis in 1-3 loco-regional ganglia): 62 cases - 23.93%;
- N2 (metastasis in 4 or more lymph nodes): 68 cases - 26.25%;

- Nx (no loco-regional ganglion invasion was determined): 46 cases, representing - 22.11%.

Correct and complete staging was performed on 212 of the 260 patients and I obtained the following results: 19 patients in stage I (8.96%), 56 stage II patients (26.41%), 85 stage III patients (40.09%), 52 Stage IV patients (24.52%).

### **3.3. Discussions**

Increased incidence rates in several Eastern and Asian countries are supposed to reflect living conditions, diet and lifestyle changes associated with Westernization, including smoking and obesity. One of the factors that could explain this difference between our study and the incidence rate at national level may be linked to an important risk factor - obesity and overweight. Obesity and total caloric intake are independent risk factors for colorectal cancer (Giovannucci E, 2002, Giovannucci E, 2003) as shown by the present studies (Singh PN, Fraser GE, 1998, Slattery ML, Potter J, Caan B et Al., 1997), and it also appears that this relationship is more prominent in males than in women (Renehan AG, Flood A, Adams KF, et al., 2012).

In the study we conducted, the patients' ages were between 37-91 years of age, with an average age of 65.53 years. The mean age of the male gender for the studied cases was 66.16 years higher than that for the female sex that was 64.43 years old. Rates are declining among adults aged 50 and older, but the number of new cases in people under 50 is increasing. Reasons for this trend are still unknown, but can be reflected by the high prevalence of obesity and poor nutrition in children, and Young adults.

From the data obtained, we note that there were no statistical differences for colon regions classified as right and left colon, the results obtained by us support the data of the studies performed globally and strengthen the tendency to increase the proportion of right colon cancers.

In the results obtained in the current study the histopathological type was the most common adenocarcinoma without further explanation, diagnosed in approximately 90% of cases, correlated with the data obtained in other histological studies.

Although several factors have been identified that have an impact on survival and the recurrence rate, none outweigh the staging in terms of importance and prognosis (Compton CC, 2002). Staging of colorectal cancer should be done using the current American TNC (tumor, node, metastasis) classification of the American Joint Committee on Cancer (Edge SB, Byrd DR, Compton CC, et al., 2010).

### **3.4. Conclusions**

We conducted an analytical and prospective study on a total of 259 patients diagnosed with colorectal carcinoma (CRC) within two years, 2014-2015.

The analysis of the distribution of colorectal carcinomas showed that the most affected decade of life was between 60-70 years, and by gender the net predominance of male patients (164 cases - 63.32%) compared to female patients (95 Cases - 36.67%) without statistical differences on age by sex.

The most common type of histopathology was adenocarcinoma without further specification, followed by mucinous adenocarcinoma, tubulo-papillary adenocarcinoma, villous adenocarcinoma, small cell adenocarcinoma, squamous cell adenocarcinoma, undifferentiated carcinoma and adenosquamous carcinoma.

Moderately differentiated tumors were the majority in all tumor types, regardless of macroscopic appearance. Mostly well-differentiated tumors were highlighted in vegetative tumors and poorly differentiated tumors were most frequently encountered in infiltrating tumors.

We found a direct increase in the potential of a lower differentiation tumor to invade one of the studied structures (vascular, perineural, lymphatic), which are negative prognostic factors in colorectal cancer.

We concluded that patients with vegetative tumors showed the most frequently without loco-regional lymph-node invasion (60.86%), on the other hand the infiltrative tumors presented in 67.85% of time loco-regional lymph-node invasion. It is also noteworthy that invasion of loco-regional nodes classified as N1 (1-3 ganglia) was most frequently encountered in vegetative and infiltrative tumors and ganglion invasion classified as N2 (> 4 ganglia) was most frequently encountered in the case of ulcerated tumors (40.47%) and the fact that vegetative tumors did not encounter N2 ganglion invasion and only in 17.23% cases of N1 invasion.

The data showed an obvious correlation between the potential for ganglion metastasis and the degree of tumor differentiation, in well differentiated tumors the patients were present in 41.02% of cases with lymph-nodes invasion, for moderately differentiated tumors in 60.52% of cases had lymph node invasion and tumors, poorly differentiated even in 80% had metastases in loco-regional lymph-nodes.

## **CHAPTER 4**

### **Immunohistochemical study of colorectal carcinomas**

#### **4.1. Materials and methods**

The immunohistochemical analysis was performed on a batch of 60 cases of colorectal adenocarcinoma that originated from the clinical and histopathological group, patients who underwent tumor resection or tumor fragments that came from an endoscopic examination.

#### **4.2. Results**

We followed the tumor angiogenesis process using immunohistochemical markers: CD31, CD105 and vascular endothelial growth factor (VEGF) expression. These may be factors of prognosis, even independent of the tumor stage.

To assess disease prognosis, we determined vascular microdensity using CD105 and CD31 marker and microvascular area.

After evaluating specific areas with all variability between individual measurements, the average area of the CD105 + -labelled vessels was significantly lower in the non-invasive tumor resection (normal colonic mucosa)  $-495.28 \pm 225.87 \mu\text{m}^2$  tissue than in the tumor invasive samples -  $2638 \pm 976.23 \mu\text{m}^2$  (t Test,  $p < 0.01$ ).

The results obtained using the CD31 marker for tumor vascularization were higher than those obtained with CD105, but were not significant ( $3150.5 \pm 890.35 \mu\text{m}^2$ ). This is normal, given that the CD31 marks both the pre-existing, mature and young ones of the neoformation.

CD31 variations between the three degrees of differentiation found are not considerable; However, the results offered by the CD31 tag are slightly lower for G1 (165.44) compared to G2 (170.63), and lower for G2 compared to G3 (172.29).

We have found, however, that the differences between the 3 degrees of differentiation for CD105 are significant, which can make CD105 a valuable tool for assessing differentiation rates in colorectal cancer. G1 - 109.7, G2 - 139.23, G3 - 166.42.

A significant difference was recorded between the three levels of stage T in the patients in our study with respect to the marker value CD105, for which we obtained the following results: T2 117.54, T3: 144.56, T4: 153.26.

For the CD31 marker we did not identify a significant difference between the three levels of the T stage encountered in the patients studied, the results being: T2 - 161.45, T3: 170.55, T4: 176.35.

Regarding the intensity of VEGF immunoexpression, we divided the cases in two categories: negative or weak positive and positive with moderate and intense intensity. Thus, the cases studied were roughly equally divided into the two categories: 33 cases - negative or low positive (55%) and 27 cases with modulated or strongly positive intensity (45%).

From the point of view of the relationship with the vascular area, the results obtained did not indicate any correlation between the area and the expressed VEGF intensity.

### **4.3. Discussions**

Angiogenesis is a complex process in the development of neoformation vessels involved in tumor proliferation and growth (Compton CC, Fielding LP, Burgart LJ, et al., 2000). The various pathways and molecules involved in angiogenesis, the formation of new blood vessels, have been studied both for the knowledge and understanding of colorectal cancer (Folkman J., 2002) and for the development of new molecular therapies (Efsthios T Pavlidis, Theodoros E Pavlidis, 2013).

Tumor tissue vascularization is higher compared to normal tissue. The process of forming new vessels from existing vessels or the process of angiogenesis, plays both the role of nutrients intake and growth factors, and as means of dissemination for tumor cells.

Our immunohistochemical CD105 staining study confirms that the vascular area in colorectal tumors is significantly higher than in the normal colonic mucosa vascular area ( $p < 0.01$ ). Positive CD105 vessels were found to be larger in number and have a significantly larger intratumoral diameter compared to normal colonic mucosa vessels, data confirming the presence of the angiogenesis process.

Both increased microvascular density and VEGF expression have been found to be independent prognostic factors independent of tumor stage, correlating with lower global survival, but especially with a shorter disease free interval.

### **4.3. Conclusions**

We tracked the process of tumor angiogenesis in colorectal cancer using immunohistochemical markers: CD31, CD105 and vascular endothelial growth factor (VEGF) expression. If CD105 marks the presence of young vessels, CD31 highlights both the pre-existing mature vessels and the neofunction vessels.

Quantification of intratumoral angiogenesis was done by assessing vascular density (VD) and microvascular area, using immunohistochemical staining for CD31 and CD105 and by evaluating vascular endothelial growth factor (VEGF) expression.

The immunohistochemical study on the process of angiogenesis in colorectal cancer represents an integration of the fundamental research and specialized medical clinic through which fundamental research succeeded in transferring to the clinic useful information that would bring therapeutic benefits and implicitly improve the prognosis of these patients.