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Polypoid and Non-Polypoid Colorectal Epithelial Neoplastic Lesions

SUMMARY OF THE DOCTORAL THESIS

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I. GENERAL PART – CURRENT STATE OF KNOWLEDGE

Colorectal cancer (CRC) is one of the **leading causes of cancer-related morbidity and mortality** worldwide, ranking third in incidence, with a continuously increasing trend [1]. In **Romania**, according to Globocan 2022 data, 13,541 new cases of colorectal carcinoma were diagnosed, placing this tumor in **the first position for incidence and second for mortality**. Most colonic adenocarcinomas arise from precursor lesions [2], either conventional colonic adenomas or serrated lesions, emphasizing the importance of early detection and treatment to prevent progression to malignancy. CRC is a complex disease that develops through the gradual accumulation of genetic and epigenetic alterations, and is one of the malignancies with the highest mutational burden [3]. CRC screening is essential for the early detection of premalignant lesions.

1. Neoplastic epithelial lesions as precursors of colorectal carcinoma

According to the World Health Organization (WHO) Classification of Tumours of the Digestive System, 2019 Edition, sessile colorectal lesions include: *hyperplastic polyps* (HP), microvesicular (HP MV) and goblet cell-rich (HP GCR) subtypes, *sessile serrated lesions* (SSL) with or without dysplasia, *traditional serrated adenoma* (TSA) and *unclassified serrated adenoma* [4].

Most SSLs are located in the right colon, are flat or slightly elevated, exceed 5 mm in size, have poorly defined margins, and a pale surface often covered with mucus. A pedunculated appearance, nodular growth, or central excavation in SSLs are usually indicators of dysplasia [5]. Only 17% of **SSLs with dysplasia** (SSLD) or malignant transformation exhibit a protruding growth. About half of the dysplastic or malignant polyps measure ≤10 mm.

The mandatory morphological feature for SSL diagnosis, according to the WHO 2019 classification, is the unequivocal presence of at least one dilated basal crypt. SSLs are characterized by a serrated architecture of the intestinal crypts, involving their entire length, basal dilatations, horizontal growth along the muscularis mucosae, and crypts with unusual shapes (inverted T, boot or anchor shapes).

The reported prevalence of SSLDs varies considerably, ranging from 3.7% to 42.9%, with an overall average of 13.9%. A low prevalence of SSLDs does not suggest that malignant transformation of these lesions is rare, but rather reflects a relatively short detection window for this intermediate stage compared to SSLs or conventional adenomas [6]. Pai et al. described four dysplasia patterns in SSLD: adenomatous, serrated, minimal deviation and not otherwise specified [7]. In most cases, multiple dysplasia patterns are present within the same lesion, which explains the heterogeneous appearance of SSLs. The majority of SSLs with not otherwise specified dysplasia and those with minimal deviation dysplasia show loss of MLH1 expression (approximately 75% of SSLDs), reflecting MLH1 hypermethylation. When minimal deviation dysplasia is suspected, it is recommended to perform immunohistochemical evaluation of MLH1 expression [7]. In the other two dysplasia patterns, MLH1 expression is preserved in most cases.

One of the early events in the development of SSL is the mutation of the BRAF gene, present in approximately 90% of cases [4]. BRAF is a proto-oncogene involved in the genesis of various types of cancer, including melanoma, thyroid cancer and CRC. The BRAF V600E mutation is the most common and leads to aberrant activation of the MAPK signaling pathway, resulting in uncontrolled cell proliferation. BRAF mutation is frequently associated with hypermethylation of CpG islands (CIMP-H), leading to the inactivation of tumor suppressor genes such as MLH1. Loss of MLH1 promotes the transition from SSL to SSLD and eventually to CRC with microsatellite instability (MSI), through the rapid accumulation of mutations. The Wnt pathway is activated in approximately 25% of SSLD cases through mutations in RNF43, APC or ZNRF3. These lesions lead to BRAF-mutant CRC, microsatellite stable (MSS) and may associate with TP53 mutations. Nuclear expression of Beta-catenin confirms the involvement of the Wnt pathway.

The two types of CRC that form through the serrated neoplasia pathway from SSL have different characteristics in terms of the immune microenvironment, response to treatment and prognosis. MSI CRC is characterized by an immunologically active tumor microenvironment, responds to checkpoint inhibitor treatment and tends to have a favorable prognosis. In contrast, MSS CRC usually presents at an advanced stage, is poorly differentiated, resistant to checkpoint inhibitors, has a higher metastatic potential and a reserved prognosis.

TSA is a serrated polyp with a risk of malignancy, much rarer, representing approximately 2% of all colorectal polyps and 1-7% of serrated polyps. TSA is most commonly located in the recto-sigmoid area, has a protruding growth, is pedunculated and may have a pinecone-like appearance. Rarely, it can be found in the right colon, especially in the flat type [8]. The distinctive features of TSA include: columnar epithelium with elongated, penciled nuclei, fine-dispersed chromatin and eosinophilic cytoplasm; serration, with the formation of epithelial spaces resembling the small intestine mucosa and ectopic crypts, small abnormal, horizontal crypts that are not anchored to the muscularis mucosae. At least two of the three characteristics mentioned above must be present for a TSA diagnosis, with at least one of them appearing on 50% of the polyp surface [9]. The first two characteristics are considered to have greater specificity.

Most TSA exhibit typical exophytic, protruding growth and a complex villous architecture. However, particular morphological variants have also been described: flat TSA, filiform TSA and mucin-rich TSA. The flat variant does not display the villous, protruding appearance typical of TSA nor ectopic crypts. However, cytoplasmic and nuclear changes, as well as the distinct serration pattern, help in the differential diagnosis with a SSL. It frequently arises from a precursor lesion (HP or SSL). The filiform variant of TSA features more elongated villous structures compared to the classical variant, with significant stromal edema at the villous tip. Mucin-rich TSA is defined by the presence of a mucinous appearance on more than 50% of the polyp's surface.

The characteristic cytological changes of the neoplastic TSA cells should not be considered dysplastic, but rather senescent changes [4]. Dysplasia, when it occurs, is of the intestinal type, similar to conventional or serrated dysplasia and the transition to the dysplastic area is abrupt. Most TSAs harbor BRAF mutations (in proximal forms) or KRAS mutations (in distal forms). Both types of mutations can induce CpG hypermethylation; however, MLH1 expression is preserved and the resulting CRC is MSS.

Colorectal **HPs** are considered benign in most cases, although rarely they can serve as precursors for other serrated polyps, which may later evolve into cancer [10]. Most HP are diminutive polyps (< 5 mm), with approximately 70% of them being located in the distal colon and rectum. They appear as discrete mucosal elevations. Up to 80% of HP MV harbor a BRAF gene mutation and half of the HP GCR mutations show a KRAS mutation. The serrated

appearance affects the upper two-thirds of the crypt length and is subtle in the case of HP GCR; no basal dilatations of the intestinal crypts are present.

The category introduced in the WHO 2019 classification, **serrated adenoma, unclassified**, includes polyps with serrated architecture and dysplasia that do not fully meet the criteria for SSL or TSA. Some tubulo-villous adenomas with epithelial serration have been termed serrated tubulo-villous adenomas and have a molecular profile intermediate between TSA and conventional adenomas. Differential diagnosis with TSA can be challenging, especially in forms with extensive dysplasia.

Serrated polyposis syndrome is the most common colorectal polyposis syndrome, with an increased risk of CRC, affecting both sexes and often diagnosed in the sixth decade of life. It is frequently underdiagnosed because it is based on a cumulative number of serrated polyps diagnosed over a lifetime (≥ 5 serrated lesions located proximal to the rectum, ≥ 5 mm, of which at least two are ≥ 10 mm, or 20 serrated lesions of any size, distributed throughout the colon, at least five of which are located proximal to the rectum). The etiology is partially understood, with potential genetic contributions (e.g., RNF43, MUTYH, GREM1). First-degree relatives have a 5-fold increased risk of CRC compared to the general population.

Conventional adenomas represent the majority of precancerous lesions associated with CRC, characterized by a dysplastic clonal proliferation of the intestinal epithelium, associated with chromosomal instability (**CIN**). They are frequently located in the distal colon, especially in the sigmoid and rectum, most of them being polypoid, under 10 mm, with a darker color than the surrounding mucosa. Histologically, from an architectural perspective, they are classified into tubular, tubulo-villous and villous types, and dysplasia is assessed as low-grade (LGD) or high-grade (HGD).

CIN, the main pathway for tumor progression, is initiated by mutations in the APC gene, followed by KRAS and later TP53 mutations, promoting cell proliferation through the activation of the Wnt and MAPK pathways. Mutations in SMAD4, PTEN, PIK3CA, and NTRK can be involved secondarily. 30% to 50% of sporadic CRCs, with hypermethylation of the MLH1 promoter, arise from conventional adenomas with KRAS mutations [4].

Advanced adenomas are defined by dimensions ≥ 10 mm, villous architecture, and/or high-grade dysplasia, and represent lesions with a high risk of malignant transformation. They are often synchronous or metachronous and require close monitoring.

2. Malignant polyps

Malignant polyps are **early forms of CRC**, corresponding to the TNM stage **pT1** and are characterized by invasion into the submucosa without involvement of the muscularis propria. The prevalence of malignant polyps in screening programs is up to 12%. The risk of malignancy is very low in diminutive polyps and increases with the lesion size. Most malignant adenomas are located in the left colon and rectum. The NICE, Kudo, and JNET classifications are valid tools for assessing submucosal invasion in colorectal polyps.

Most of them are **adenocarcinomas NOS** (not otherwise specified), but rare subtypes may exist. Grading is done based on glandular differentiation (low vs. high grade). Risk factors for metastasis or local recurrence include poorly differentiated tumors, deep submucosal invasion (Sm3 or Haggit 4), lymphatic or vascular invasion, tumor budding and a positive resection margin, with less than 1 mm from the tumor. The decision to perform surgery in a malignant polyp must be made by a multidisciplinary team after analyzing all clinical and pathological aspects. In the absence of high-risk factors, endoscopic resection is considered curative, with endoscopic monitoring starting 3–6 months after the polyp removal.

Pseudoinvasion is frequently encountered in large pedunculated polyps in the sigmoid colon, that have been exposed to mechanical trauma. It consists of the migration of dysplastic glands into the submucosa, without infiltrative characteristics. The presence of the lamina propria around the dysplastic glands, with atypia similar to that in the mucosa, hemosiderin deposits and mucin lakes in the submucosa are features identified in pseudoinvasion.

Screening prevents CRC by identifying and removing precursor lesions, significantly contributing to a reduction in the disease's mortality and incidence by up to 50% [12]. In Romania, the ROCCAS program was initiated in 2019 and targeted the population aged between 50 and 74 years, without high-risk factors, being implemented in four regions as a pilot project. The program achieved a high acceptance rate for the FIT test and colonoscopy, demonstrating the feasibility of expanding it nationwide. The main goal of ROCCAS was to develop a strong infrastructure for the continuous and effective implementation of screening, an essential step in a country where CRC remains one of the leading causes of cancer-related mortality.

II. PERSONAL CONTRIBUTIONS

3. Working hypothesis and general objectives

One of the research hypotheses is that two-photon excitation microscopy (TPEM), compared to conventional optical microscopy, provides superior analysis of collagen deposits in the desmoplastic reaction in malignant polyps. This technology can offer insights into the tumor's interaction with the extracellular matrix and facilitate differentiation between real invasion and pseudoinvasion, a critical aspect in the post-polypectomy management of the patient.

Another working hypothesis is that SSLs exhibit a different biological and evolutionary behavior compared to conventional adenomas. The immunohistochemical study of these lesions aims to identify markers that predict the different behavior of these lesions, including the risk of malignancy. The third hypothesis is that the presence of Paneth cells (PC) in colonic adenomas could be associated with histological features suggestive of an increased risk of progression to adenocarcinoma, by supporting a cellular niche that favors proliferation.

The general objectives include demographic and histological characterization of malignant polyps, SSL and conventional adenomas, analysis of the risk of malignancy, identification of predictive markers through immunohistochemistry and evaluation of the tumor immune microenvironment in SSL. Additionally, advanced microscopy methods will be compared to conventional methods for more precise diagnosis.

4. General methodology of the research

The studies were conducted with the approval of the Ethics Committee of the "Dr. Carol Davila" University Emergency Military Hospital in Bucharest, as well as the Ethics Committee of the "Victor Babeș" National Institute for Research and Development in Pathology and Biomedical Sciences (INCD "Victor Babeș") in Bucharest. All stages of the research were carried out in compliance with ethical standards. Data were extracted from the patients' electronic records, including demographic information, medical history and endoscopic characteristics of the identified lesions.

Biological samples were processed using standard laboratory procedures, fixed in formalin, dehydrated and embedded in paraffin, then stained with hematoxylin and eosin. For immunohistochemical studies, a wide range of antibodies were used (MLH1, p53, CD44, CD3, CD8, MUC2, MUC5AC, MUC6, Ki-67, Chromogranin, and p63), with automated processing on Dako and Ventana systems. The protocol included deparaffinization, slide pre-treatment, incubation with primary antibodies, detection, counterstaining and final mounting.

Microscopic evaluation was performed using optical microscopy, and for some cases, two-photon excitation microscopy (TPEM) was used, an advanced technique for detailed analysis of collagen fibers in lesions with invasion versus pseudoinvasion. These images provided additional insights into the tumor-extracellular matrix interaction.

Data were centralized in Microsoft Excel and statistically analyzed using IBM SPSS and GraphPad Prism, depending on the data type and the objectives of each study.

5. Histopathological and immunohistochemical profile of colonic SSL (Study I)

In this study, I analyzed 45 SSLs, aiming to evaluate their endoscopic, histopathological, and immunohistochemical characteristics in order to identify the differences between SSL with and without dysplasia and to better understand their risk of malignant transformation. The diagnosis was made according to the WHO 2019 criteria, based exclusively on polypectomy specimens, which was the main inclusion criterion for the study group.

Results

A slight predominance of the **female gender** (57.78%) was observed in the patient group included in the study, with ages ranging from 37 to 80 years and a mean age of 61.51 years. The most frequent age group was 50–59 years.

The majority of SSLs (77.78%) were located in the **right colon**, specifically in the ascending colon (53.33%). The average size of the lesions was **11.24 ± 5.6 mm**, with a range between 5 mm and 25 mm. Although no statistically significant difference was found between the sizes of SSL with and without dysplasia, I observed that **all lesions ≥ 20 mm** were **dysplastic**, and **50% of SSLD lesions were less than 10 mm**. The association between size and the presence of dysplasia was statistically significant ($p = 0.721$, Fisher's test).

During colonoscopies, synchronous lesions were frequently identified, most of which were LGD tubular adenomas (48.14% of patients with SSL and 77.77% of those with SSLD), advanced adenomas (8.88%), SSLs (15.55%), and TSA (2.22%).

Most SSLs were described endoscopically as sessile or flat, with only one dysplastic lesion having a **pedunculated** appearance. In 31.11% of cases, the resection was fragmented, and the integrity of the resection could not be fully assessed, while in 3 of the en block resected lesions, positive lateral margins were observed.

Among the neoplastic lesions included in the study, 18 showed dysplasia: 15 SSL with LGD, two with HGD, and one intramucosal adenocarcinoma (pTis). These were divided into two groups: SSL with LGD and SSL with HGD/intramucosal adenocarcinoma. Although there is invasion limited to the mucosa, the absence of lymphatic vessels in the lamina propria means that this lesion does not present a risk of lymph node metastasis.

A statistically significant difference ($p<0.001$) was observed between the mean age of patients with SSL (57.48 ± 1.77 years) and those with SSLD (67.56 ± 1.66 years). However, no significant differences were found between the age of patients with LGD and those with HGD ($p = 0.654$). No significant associations were observed between gender and dysplasia ($p = 0.712$) or between tumor location and dysplasia ($p = 0.721$).

All lesions met the diagnostic criterion for SSL, namely unequivocal basal crypt dilation. Although this feature was evident in most cases, for two small polyps (under 10 mm), additional sections were made to differentiate them from HP. The presence of gastric-type mucosecretory cells at the base of the crypts was observed in all cases. Crypt herniation was identified in 13.3% of cases, but no significant association with dysplasia or lesion location was found. Another notable feature was the presence of lymphoglandular complexes. The main architectural changes observed in dysplastic areas included: reduced serration, branching of crypts and glandular dilation.

To evaluate the proliferative compartment of SSLs, I used **anti-Ki67 antibodies**. In lesions without dysplasia, positive cells were located in the lower third of the crypts, with a discontinuous and asymmetric distribution. In dysplastic lesions, positivity was extended along the entire length of the crypts, including the surface epithelium, with a significantly higher density of positive cells. In the study, none of the SSLs without dysplasia showed aberrant

reactions of the MLH1 and p53 proteins. In contrast, **27.77%** of the dysplastic lesions had **loss of MLH1** expression and **11.11%** showed a **mutant p53 immunohistochemical pattern**.

I evaluated the expression of mucins using specific immunohistochemical markers, such as **anti-MUC2, MUC5AC, and MUC6 antibodies**. MUC2 and MUC5AC were positive in all SSL, but in areas with HGD, a significant reduction in the expression of mucins was observed. Comparing MUC2 expression with MUC5AC, I found variability in the intensity and distribution of MUC5AC. MUC6 was expressed in 66.66% of SSLs, but its intensity was lower compared to MUC2 and MUC5AC markers. No differences were identified regarding mucin expression between lesions in the left colon and those in the right colon.

I used **anti-CD44 antibodies** to analyze the expression of this protein in normal colonic mucosa and in SSLs and SSLDs. CD44 is a glycoprotein located on the cell surface, expressed in numerous cells, playing a crucial role in processes such as cell adhesion, differentiation, migration and signaling. In normal colonic mucosa, CD44 expression was limited to the base of the crypts. In serrated areas without dysplasia, CD44 positivity was observed in the lower half of the crypts. Dysplasia was associated with overexpression of the CD44 protein. In 90% of SSLD cases with preserved MLH1 expression, the CD44 reaction was weak to moderate in intensity and extended into the upper half of the crypts. MLH1-deficient dysplastic cells showed intense expression of CD44, extending to the surface epithelium. A significant correlation was observed between CD44 positivity and loss of MLH1, as well as between CD44 expression and Ki67 ($p<0.001$, Fisher's test).

To evaluate the immune microenvironment, I used **CD3 and CD8 markers**, specific to T lymphocytes, which are essential in the antitumor immune response. I identified three distinct patterns of CD8-positive intraepithelial T lymphocyte density:

- ✓ A maximum of 1-2 intraepithelial lymphocytes per 100 epithelial cells in dysplastic areas with preserved MLH1 expression;
- ✓ Up to 10 intraepithelial lymphocytes per 100 epithelial cells in serrated areas without dysplasia;
- ✓ Up to 14 intraepithelial lymphocytes per 100 epithelial cells in dysplastic areas with MLH1 deficiency.

I considered a threshold of 10 intraepithelial lymphocytes to differentiate the number of lymphocytes in non-dysplastic serrated areas from those in dysplastic areas with lost MLH1

expression. A statistically significant association was observed between the number of intraepithelial CD8+ T lymphocytes and epithelial dysplasia, with or without MLH1 expression loss ($p<0.001$, Fisher's test).

I evaluated the distribution of neuroendocrine cells in normal colonic mucosa and in SSL using chromogranin. Compared to adjacent normal mucosa, I observed a general reduction in the number of neuroendocrine cells in SSL. In certain areas, these cells were completely absent, while in others, they were present in low numbers, particularly in regions with basal architectural distortion.

6. Evaluation of malignant colorectal polyps – the role of Two-photon excitation microscopy in tumor invasion diagnosis (Study II)

In this study, I evaluated the clinicopathological characteristics of malignant colorectal polyps and characterized the stromal changes that occur in malignant polyps using a complementary nonlinear optical imaging technique (TPEM). The study group included 58 patients, 53 of whom were histopathologically diagnosed with malignant colorectal polyps, while for the remaining five patients, the diagnosis of polyp with pseudoinvasion was established. For image analysis using TPEM, I selected a total of 14 polyps, nine with a diagnosis of malignancy and the remaining lesions, colonic adenomas, with pseudoinvasion features.

This part of the study was conducted through a collaboration between the Department of Pathology at the "Dr. Carol Davila" University Emergency Military Hospital in Bucharest and the Microscopy, Microanalysis and Information Processing Center at the National University of Science and Technology Politehnica, in Bucharest. TPEM utilizes two nonlinear optical contrast mechanisms – second harmonic generation (SHG) microscopy and two-photon excited fluorescence (TPEF). Slides were scanned with an Aperio LV1 IVD scanner at 20x and regions of interest (ROIs) were examined using a Leica TCS SP confocal microscope. The TPEF signals originated from regions containing eosin, while SHG signals came from collagen fibers.

Four parameters associated with the distribution of pixel intensities in SHG images were calculated: **Mean, Standard Deviation, Skewness** and **Kurtosis**. Pixels in the SHG image that

exceeded a threshold value were counted, providing the total collagen area ratio (**TC-ratio**), calculated as the ratio between this count and the total image area. The set of parameters derived from the **Gray-Level Co-occurrence Matrix (GLCM)** is very useful for describing the spatial relationships between pixel intensities in an image: **Contrast, Homogeneity, Energy, Entropy and Correlation.** SHG images were used to evaluate the orientation of collagen fibers by analyzing the Fast Fourier Transform (FFT) with a custom script written in ImageJ. The images were initially binarized and then fitted with an ellipse. A collagen orientation index (N) was calculated based on the minor (S) and major (L) axis lengths of the ellipse: $N = 1 - S/L$ [14]. An alternative approach to objectively quantify the organization in an image involves fractal analysis. SHG images were analyzed after thresholding by the Triangle method, i.e., in binary format, using an ImageJ plugin (e.g., Fractal box count) to calculate the fractal dimension.

Results

All 53 malignant polyps were conventional adenomas, with the malignant component predominantly consisting of low-grade adenocarcinoma, NOS (94.33%). The remaining cases were mucinous adenocarcinoma and signet-ring cell adenocarcinoma. A predominance of male gender was observed (64%). The patients' ages ranged from 51 to 87 years, with a mean age of 66.5 years. The majority of patients were in the age group 61-70 years (51%). Most malignant polyps were located in the sigmoid colon (52.8%), followed by the rectum (22.7%), ascending colon (9.4%), descending colon (7.5%), transverse colon and cecum (each 3.8%).

The size of the malignant polyps ranged from 8 mm to 60 mm, with a mean size of 25.36 mm. Regarding morphology, 22 polyps were pedunculated, 12 were semipedunculated, 10 were sessile. These information for 9 polyps was not specified. Endoscopic resection was performed in 48 cases, while 5 sessile or flat polyps were surgically resected. Synchronous lesions were frequently identified, including 25 patients with advanced adenomas and 2 with concomitant adenocarcinoma.

Risk factors for metastasis and local recurrence were evaluated, including lymphovascular invasion (13.2%), poorly differentiated malignant component (5.67%), Haggit level 4 invasion (1.9%), positive resection margins (9.4%), and tumor budding, Bd1 (39.6%). The decision for surgical intervention was made in six cases, considering the patient's age, overall health status, comorbidities, associated risks, surgical tolerance and disease progression.

After surgery, one case of local recurrence and one case of lymph node metastasis were identified.

For the analysis of collagen distribution and organization, 88 images from invasion areas of malignant polyps and 65 images from pseudoinvasion areas, both with significant collagen content, were selected from SHG images obtained from areas of 1 x 1 mm^2.

All calculated parameters, including Mean, Standard Deviation, Skewness, Kurtosis, TC-ratio, Orientation, Homogeneity, Energy, Entropy and Contrast, detected **statistically significant differences** between SHG images obtained from malignant polyps and polyps with pseudoinvasion ($p < 0.05$, $p < 0.01$, $p < 0.0001$), except for the Fractal Dimension. The collagen orientation index (N) calculated indicates a **higher index in malignant polyps**, suggesting a more ordered and uniform orientation of collagen fibers in these areas compared to pseudoinvasion.

7. Morphological particularities of conventional colorectal adenomas (Study III)

Conventional adenomas are the most common precursor lesions for CRC and they may exhibit various morphological changes that can influence their clinical behavior. These changes include the presence of Paneth cells (PC), clear cells, "signet-ring" cells or squamous metaplasia, all of which may affect clinical outcomes. Other characteristics, such as the formation of ectopic crypts and cytoplasmic eosinophilia, can complicate the differentiation of conventional adenomas from other pathological entities, such as TSA. This study aims to contribute to the understanding of colorectal adenomas' diversity through morphological analysis and the clinical implications of these lesions.

Results

We analyzed 273 colorectal adenomas from 209 patients (94 women and 115 men), with ages ranging from 25 to 88 years. The majority of patients were in the age groups 61-70 years (36.84%), followed by 71-80 years (27.75%) and 51-60 years (23.44%). 7.69% of adenomas were diagnosed in patients under 50 years old. Most of the adenomas were located in the left side of the colon, particularly in the sigmoid colon (34.07%). In 10.62% of cases, the location data was unavailable.

Endoscopically, Paris classification was used in 77.65% of cases, while NICE, Kudo or JNET classifications were applied in 30.40% of cases. According to Paris classification, 58 pedunculated polyps, 75 sessile polyps, 60 semipedunculated polyps and 19 flat polyps were examined.

The size of the conventional adenomas ranged from 2 mm to 45 mm, with 73.98% of lesions being subcentimeter. 26.01% were larger than 10 mm, being classified as advanced adenomas. The architecture of the examined adenomas was predominantly tubular in 85.35% of cases, followed by tubulo-villous (13.92%) and villous (0.73%). The majority of adenomas presented LGD in 83.53% of cases, while 12.82% had HGD. In 3.66% of cases, polyps were malignant, showing proliferation of low-grade adenocarcinoma, NOS. 90% of the malignant polyps had a tubulo-villous or predominantly villous architecture. Additionally, 6.57% of tubular adenomas and 43.24% of villous adenomas showed HGD.

PCs [15] were identified in 47 conventional colorectal adenomas (17.22%). Most adenomas containing PCs were located in the sigmoid colon (29.79%), followed by the transverse colon (23.40%) and the ascending colon (17.02%). In 8.51% of cases, the lesion location was not available.

61.70% of these adenomas were diagnosed in men. 18 patients had synchronous adenomas (38.29%) and one of them had a synchronous adenocarcinoma. 40 adenomas (85.10%) showed LGD and five (10.63%) showed HGD. Two malignant polyps contained PCs (4.27%) within the adenomatous component. PCs were not found in the malignant component.

I identified a case of **squamous metaplasia** in an adenoma located in the left colon, pedunculated, with HGD. I used the anti-p63 antibody in the immunohistochemical analysis, which showed absent expression in the squamous metaplastic area. Additionally, a 64-year-old patient, diagnosed with a tubulo-villous adenoma with HGD, presented an area of **metaplasia with clear cells**. Two adenomas showed a predominance of goblet cells and were located in the left colon, with an average size of 34 mm and LGD. Ectopic crypts were identified in 45 cases (16.48%) and were associated with lesions ≥ 10 mm or with a villous component. Another change was cytoplasmic eosinophilia, which, along with ectopic crypts, is characteristic of conventional serrated adenomas. This was identified in 7.69% of the cases studied.

8. Conclusions and personal contributions

The objective of this doctoral thesis is to study the main precursors of CRC, specifically conventional adenomas and SSLs, to better understand the behavior of these neoplastic lesions. The results obtained have confirmed existing findings in the literature while also introducing new insights. The studies conducted focused on three major research directions.

Study I was dedicated to the evaluation of colorectal SSLs. The main conclusions were:

- ✓ 50% of SSLDs had sizes between 5 and 9 mm, highlighting the need for rigorous clinical and endoscopic evaluation.
- ✓ All lesions with a size ≥ 20 mm showed dysplasia, with a statistically significant correlation between lesion size and dysplasia.
- ✓ Although the average age of patients with SSL without dysplasia is about 10 years younger than that of patients with dysplastic lesions, no statistically significant difference was observed between the average age at the time of diagnosis for LGD and HGD, suggesting that the evolution of these lesions is rapid once they develop dysplasia.
- ✓ In the presence of dysplasia, a common feature of all the examined SSLs was a significant reduction in epithelial serration, indicating that careful examination of the margins is crucial.
- ✓ A significant proportion (27.77%) of SSLDs showed loss of MLH1 expression, highlighting the role of MSI in the serrated pathway of carcinogenesis.
- ✓ 11.11% of SSLDs with LGD and HGD displayed a mutant immunohistochemical pattern for p53, raising the question of whether the TP53 mutation might be an earlier event in SSLDs compared to conventional adenomas.
- ✓ Reduction in the expression of MUC2 and MUC5 in regions with HGD/intramucosal adenocarcinoma suggests a loss of the natural mucosal protection mechanism conferred by mucins, thereby facilitating the progression of the lesion towards malignancy.
- ✓ SSLDs with MSI exhibit distinct immunological characteristics, demonstrating intraepithelial lymphocytic infiltration with CD8+ cytotoxic T lymphocytes in regions of MLH1-deficient dysplasia.

- ✓ CD44, a biomarker of tumor stem cells, was overexpressed in all cases and the expression being was more intense in MLH1-deficient areas compared to those showing preserved MLH1 expression.

Despite the limitations of the study, related to the small number of cases, I obtained several significant results that will serve as a foundation for further research. This is the first study to raise the issue of a possible association between CD44 overexpression and MLH1 function loss, which could explain a faster progression of these lesions towards malignancy.

In **Study II**, I analyzed the main clinico-demographic characteristics of malignant polyps, which were histopathologically confirmed as conventional adenomas. In most cases, the malignant component was represented by adenocarcinoma NOS, but I also encountered rarer variants of adenocarcinoma, such as mucinous carcinoma or signet-ring cell carcinoma. The malignant polyps were more frequently diagnosed in the left colon, especially among men, with an average age at diagnosis of 66.58 years. I assessed the risk factors for locoregional metastasis and recurrence.

To differentiate pseudoinvasion from invasion in malignant polyps, I used a special TPEM technique, which allowed both quantitative and qualitative analysis of collagen in the intra- and peritumoral regions. This technique had previously been used to evaluate stromal changes in malignant epithelial tumors (such as thyroid, skin, breast, gastric, pancreatic and CRC), but for the first time it was employed to differentiate between pseudoinvasion and invasion in malignant polyps, offering a unique and superior view of the tumoral architecture and stromal behavior in early stages of CRC.

For all the calculated parameters (Mean, Standard Deviation, Skewness, Kurtosis, TC-ratio, Contrast, Homogeneity, Energy, Entropy and Correlation), significant statistical differences were observed between the SHG images obtained from malignized polyps and those with pseudoinvasion, except for the fractal dimension.

The results obtained could open new perspectives for the integration of machine learning and deep learning technologies in CRC diagnosis, combined with the advanced TPEM technique. This approach could significantly improve diagnostic accuracy.

In **Study III**, I evaluated colorectal conventional adenomas from a clinico-demographic and morphological perspective. One of the significant findings was the identification of a considerable percentage of colonic adenomas diagnosed in patients under the age of 50 (7.69%).

This raises the question of whether the recommended age for starting CRC screening should be revised, considering that these adenomas can also occur in younger patients. Most adenomas are located in the distal colon and their size tends to increase as they develop HGD or become malignant. However, in two studied cohorts (Study II and III), I encountered two malignant polyps with diameters under 10 mm, emphasizing the importance of careful evaluation of small polyps. This observation demonstrates that size is not always a sufficient indicator of malignancy risk and that even small polyps may have malignant potential.

I examined adenomas for the presence of PCs, clear cells, goblet cells, squamous metaplasia, ectopic crypts and cytoplasmic eosinophilia and assessed potential correlations between these features and the clinico-demographic characteristics of the patients. Clear cell metaplasia and squamous metaplasia were found in isolation in patients with HGD. Due to the limited number of reported cases of clear cell metaplasia in adenomas, the significance of this change is not yet fully understood. Additionally, ectopic crypts and eosinophilic metaplasia were frequently encountered, in a focal pattern. Ectopic crypts were associated with adenomas over 10 mm in size, with a tubulo-villous architecture.

PCs were frequently identified (17.22%), particularly in sigmoid colon adenomas with LGD, in men and in patients with synchronous adenomas (38.29%). These cells were absent in the malignant component, suggesting that PC may be more involved in the early stages of carcinogenesis.

These findings contribute to a better understanding of the biological processes involved in the development of CRC and may open new perspectives for improving prevention, diagnosis, and treatment strategies for this pathology. Further research in this direction is essential to improve the prognosis of CRC patients and develop personalized therapeutic approach.

Selective bibliography

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List of scientific publications

1. **Florea MA**, Becheanu G, Niculae A, Dobre M, Costache M. Immunohistochemical insights into the pathogenesis of colonic sessile serrated lesions. *Archive of Clinical Cases*, 2025, *12*(1), 22-28. (Chapter 5)

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2. **Florea MA**, Eftimie LG, Glogojeanu RR, Hristu R, Stanciu GA, Costache M. Imaging of colorectal adenomas with pseudoinvasion and malignant polyps using two-photon excitation microscopy. Front Oncol. 2024 Jun 14;14:1394493. doi: 10.3389/fonc.2024.1394493. PMID: 38947893; PMCID: PMC11211392. (Chapter 6)

https://www.frontiersin.org/journals/oncology/articles/10.3389/fonc.2024.1394493

3. **Florea MA**, Eftimie LG, Enea D, Becheanu G, Costache M. Paneth Cells: A Comprehensive Review of Their Role, Prevalence and Molecular Mechanisms in Colorectal Neoplastic Lesions. R. J. Mil. Med. 2025, 128(2): 166-170. (Chapter 7)

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