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DOCTORAL THESIS
- ABSTRACT-

CONTRIBUTIONS TO THE ASSESSMENT OF CLASSIC AND MODERN PROGNOSTIC FACTORS IN COLORECTAL CARCINOMA

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INTRODUCTION

CRC remains to be a public health issue. It is placed on the first place amongst cancers in the gastrointestinal tract (1)(2) and generally represents the second cause of death by cancer (3). The CRC incidence is going up for both sexes, it represents the third most frequent type of cancer in men, after bronchopulmonary cancer and prostate cancer, and in women, it is the second after mammary cancer (3)(4).

There are developed countries where by the reduction of the risk factors action, the implementation of the screening programs, the improvement of the imaging and surgical techniques which registered a decrease of the CRC incidence and of the mortality due to such disease (5). Despite all this, In Australia and the United States of America (USA), due to unknown reasons, the CRC incidence rates at young adults, under 50, are growing (5)(6). IN the countries with few resources, CRC shows increase rates of the incidence and mortality (7)(8).

According to the Globocan statistics for Romania, in 2018, the CRC incidence was 13.3% (11.076 new cases), being the second cancer as incidence both in men and in women, and the third as mortality for both sexes (8). In our country, due to late symptomatology, the patients visit the physician most often in advanced stages of the disease (8)(9). A diagnosis of the disease in advanced stage is tightly connected a low socio-economic status, the limited access to comprehensive medical services – including diagnosis and treatment, to the lack of information and lack of education for health some patients may suffer from. A different cause of late CRC diagnosis is represented by the lack of screening programs for the population and of individual undergoing high risk, which may detect and remove the precursor lesions and/or the tumor in early stages.

A major preoccupation in the field of oncologic research these days is held by the identification of the prognostic indicators, which offer an idea on the seriousness of the disease. The TNM (Tumor, Node, Metastasis) Stage, assessed according to the system recommended by The American Joint Committee on Cancer/ The Union for International Cancer Control (AJCC/UICC), represents the most important prognostic indicator for CRC, as it guides therapeutic options within clinical practice (1). This classification synthesizes the data on the local extension of the tumor (T), the status of regional lymph nodes (N) and the occurrence of distant metastases (M) (10). However, the value of such parameter is questionable, seeing that there are studies which show an appreciable variability of the clinical course for the CRC patients found in the same stage of the disease (11). Additionally, some CRC cases, stage 1/2 undergo aggressive course, while a part of CRC patients, stage 3, undergo a favorable course (12). Therefore, the detection of factors to improve the selection of CRC patients, for proper therapy, and avoiding to overtreat or treat defectively some cases, is a necessity.

In that regard, there is worldwide a deep concern to identify, validate and implement new parameters to allow the anticipation of the CRC patients course and the prediction of the response to treatment.

MOTIVATION AND OBJECTIVES OF THE DOCTORAL STUDY

I have carried out this doctoral study taking into account that the prognostic and predictive indicators identified up to now fail to offer enough data in order to accurately stratify the CRC patients in risk groups with adapted treatment. We've started from the premise that by a more comprehensive and complete histopathological interpretation of biopsies and surgical parts, we will be able to identify new parameters/markers to predict the risk of recurrence or of aggressive course of the disease. Therefore, I planned to analyze, besides classic prognostic indicators, a series of less known, and/or accounted for, morphologic parameters in CRC.

In view of the things above, **the motivation** to carry out this doctoral study was to *improve the therapeutic management* of CRC patients.

The purpose of this doctoral thesis was to *assess the classic and modern prognostic indicators in CRC.*

In order to fulfil such purpose, we set out the following **main objectives**:

- to shape a profile of the CRC patients, of their tumors, that is, diagnosed in a reference facility in the west part of Romania;
- to assess new morphologic prognostic indicators, which may improve the therapeutic management of the CRC patients, by using cheap methods, easy to implement in daily practice in pathologic anatomy labs.

In particular, in order to fulfil the objectives of this doctoral study, we carried out the following categories of tests:

1. ***Demographic data analysis*** *and* ***assessment of classic clinical and morphological parameters in CRC***, all included in a retrospective long-term study extended over a period of 10 years, which encompassed the CRC cases diagnosed according to surgical resection parts at the "Pius Brînzeu" Emergency County Clinical Hospital of Timișoara (SCJUPBT).
2. ***The assessment of new morphologic parameters in CRC***, in order to assess their prognostic significance:
   - ***assessment of classic morphologic parameters*** (ulceration and tumoral necrosis, TNM AJCC stage of the disease, PNI) ***additional*** to the usual and to modern morphologic parameters (TILs, configuration of the invasion front of the tumor) in CRC;
   - ***TB assessment*** by two categories of tests:
     - TB assessment in order to standardize the assessment method, to check inter-observer variability and that of the applicability of such method in daily practice, on a lot of CRC cases which underwent robotic surgery, on scanned slides, HE and IHC colored;
     - TB assessment in CRC, in order to determine the prognostic significance of such new parameter and to compare the grading system based on TB quantification (GBd – Buds Grade) with the classical histologic grading system (the WHO degree);
   - ***PDCs assessment*** from the perspective of appreciating the prognostic significance of such new parameter and of comparing the grading system based on PDCs quantification (PDCs-G) to the classic histologic grading system (WHO degree).

This doctoral thesis consists of a **general part** and a **specific part**, dedicated to my own contributions. The general part of the thesis, structured on three chapters, shows data on the current stage of knowledge related to CRC, after an analysis of the many bibliographic reference sources in the field.

The **first chapter of the capitol general part** approaches the clinical and epidemiological, etiopathogenic particularities and underlined the importance of the risk factors in CRC. Epidemiologic studies show that the overall CRC incidence is higher in men (6). It's also been shown that CRC with early onset is advanced stage and has adverse pathologic features related to a risk of premature morbidity and death (13)(14). The CRC risk is higher after 40 years old, and doubles with each and every decade after 50-55 years old and continues to increase exponentially (15). The basic mechanisms and the risk factors for the emergence of such wounds / lesions are not fully understood, and therefore additional researches in the field are justified. There are epidemiologic studies which suggest that this disease may come from comprehensive action of risk factors (epigenetic mechanisms) with the genetic sublayer (16), but most of the CRC are sporadic and not family related (17). The risk of CRC emergence at persons without CRC personal/family history and aged over 50 is 5-6%. It gets higher up to 20% in case of close relatives suffering from CRC and reaches 80-100% in case of hereditary syndromes (18). Familial Adenomatous Polyposis – FAP and the Lynch syndrome - Hereditary nonpolyposis colorectal cancer – HNPCC are the most frequent of all syndromes undergoing family transmission, but altogether these two diseases

only represent more or less 3-5% of the CRC cases (19). The neoplastic transformation which progresses according to the conventional pattern of carcinogenesis, the so-called adenoma-carcinoma sequence, takes the tubular, villous or tubulovillous adenoma as precursor lesion (20). Two mechanisms of carcinogenesis are described at a molecular level: chromosomal instability (CIN) - 85% of the cases, microsatellite instability (MSI) - 15% of the cases (21). Unlike the conventional way of adenoma-carcinoma transformation, an alternative way (the serrated way) has been documented for ten years, by identifying the adenomas /serrated polyps as precursor lesions (22).

The **second chapter of the general part** comprises data on the localization, clinical manifestations, macroscopic, microscopic aspects and staging in CRC. The macroscopic aspects of the CRC are variable, the ulcerative-infiltrating form is most common which can affect circumferentially the lumen of the intestine and causes variable stenosis degrees (up to the occurrence of bowel occlusion). In terms of the histologic tumor subtype, the most cases of CRC (90%) are adenocarcinomas (ADK) and most of the studies only concern them (1). By definition, ADK are tumors which invade the submucous, by the muscularis mucosae. Although most of the cases are diagnosed as ADK NOS (conventional ADK), several histopathologic subtypes can be distinguished, which include distinct clinical and molecular features.

The prognostic indicators for CRC, both classic and modern, have been analyzed, depending on the data in the literature, in the **third chapter of the general part**. Although the parameters which determine the pathologic stage of the disease - pTNM are the truest indicators of postoperative evolution, and other clinical, histological and/or molecular parameters, irrespective of the stage, can influence the prognostic. Amongst the classic parameters, long studied and validated, we will list the following: the histologic type of the tumor, the tumoral degree of differentiation (G), the lymphovascular invasion (LVI) and the perineural invasion (PNI) (1). Of concern during the times has also been a series of parameters which can be interpreted on slides in typical staining, hematoxylin-eosin (HE) and immunohistochemical (IHC), which makes it easier to assess and more accessible than molecular markers, but most of them still need validation and standardization in order to be included in the histopathologic report. Amongst the modern prognostic indicators, we shall mention tumor budding (TB) (23), the groups of poorly differentiated cells clusters at the level of the tumor invasion front (PDCs) (24), the tumor border configuration/invasion pattern (25), the tumor infiltrating lymphocytes (TILs) (26), lymph node micro metastases (27), as parameters which may complete and even replace some classic prognostic indicators for CRC.

1. ASSESSMENT OF DEMOGRAPHIC AND CLINICAL MORPHOLOGIC PARAMETERS BASED ON THE PROFILE OF PATIENTS/ COLORECTAL MALIGNANT TUMORS IN THE WEST PART OF ROMANIA

The first chapter of the specific part was intended to analyze CRC from the perspective of demographic aspects and of the main clinical and morphological indicators, with a view to ***shape a profile of the CRC patients***, and ***their tumors,*** diagnosed in a reference hospital in the west part of Romania, an identifying possible particularities related to the geographical area and exposure to certain risk factors. In this respect, we followed the development of a database in order to assess the distribution by years and the main clinical and morphologic features of the CRC as well as the assessment of the classic prognostic indicators (age and sex of the patients, tumor localization, pT parameter, pN parameter, pM parameter and LVI), by their multi-variable analysis, depending on the histologic type of CRC.

The database included all the CRC patients diagnosed as result of surgical resection parts, for the period January 2009 – December 2018, in the Surgical Clinics of the “Pius Brînzeu” Emergency County Clinical Hospital in Timisoara (SCJUPBT). In the west part of

Romania, SCJUPBT represents a reference medical facility, with high addressability, which deliver medical services at high standards in the digestive oncologic scope. For the lot of **1885 CRC cases** included in the study, the clinical data were collected from the biopsy material cover note and from the care report forms, and the histopathological parameters – from the histopathological notes in the databases of the Pathological Anatomy Service (SAP) of SCJUPBT.

Inclusion criteria in the study – all consecutive CRC cases, diagnosed by the histopathological examination of the surgical resection parts – radical and standard, with regional lymphadenectomy.

Criteria for exclusion from the study lot:

- patients with different types of cancer, but carcinomas;
- patients with CRC diagnosis set according to endobiopsy or polypectomy specimens;
- patients with tumor recurrences;
- patients which benefitted from neoadjuvant radio-chemotherapy treatment.

The CRC diagnosis was established after standard histopathological processing of the surgical resection part, within SAP of SCJUPBT.

In order to appreciate the histologic subtype and the differentiation degree, We have used the classifications issued by the WHO to frame and classify the tumors of the digestive system, and the pathological staging classification was carried out in accordance to the pTNM AJCC/UICC system (the editions in force the moment of the case assessment) (10)(28).

We have selected out of the histopathological reports and entered in Excel the following demographic and clinical morphological parameters: the patients' age, the patients' sex, the year when diagnosed, the paraffin blocks related number, the CRC localization, the histologic type of the tumor, we have documented the synchronous double/multiple tumors, the differentiation degree of the tumor, the presence of distant metastases – pathologically documented (pM1); the presence/absence of the lymphovascular invasion (LVI+/LVI-).

The collected parameters have been analyzed statistically by using Microsoft Excel, Graph Pad Prism v6 and IBM SPSS v20. We have used the *Student t test*, *Chi – square* or *Fisher's exact test*, in order to analyze the correlations and/or differentiations amongst the clinical and pathological indicators. The ***p*** value resulted was considered statistically significant if lower than **0.05**.

In order to fulfill my objectives in my doctoral thesis, I acted in compliance with the principles of the Declaration from Helsinki of ethical principles for medical research. Every and each patient signed an informed consent form (Annex 4 to the Application Norms *of Law 104/2003)*, which allows the use in medical studies of the biological products sampled and the use for teaching/scientific purposes the photos of tissues or organs.

Conclusions. From the perspective of analyzing the distribution by years to the lot of 1885 CRC cases, we noticed the increase of the number of CRC cases along the time interval analyzed (2009-2018). CRC were more frequently diagnosed at patients over 60 (69.45%), more frequently in the seventh decade of life, at both sexes. Colorectal carcinomas were diagnosed mainly at the male sex (58.25%), and as regarding the tumor topography, they were more frequently identified at the left colon (41.44%). Depending on the histologic tumor type, most of the CRCs were ADK NOS or conventional type (88.96%), followed by mucinous ADK (10.54%), while the subtypes of signet ring cell carcinoma and medullary carcinoma were rarer: 0.39%, and 0.11%, respectively.

Colorectal synchronous double tumors occurred at 73 cases (4%), and were mainly placed at the level of the left colon (47.94%) and diagnosed more frequently at male patients (65.76%). Most of the synchronous tumors were ADK NOS (73.97%), deeply invasive in the intestinal wall (pT3-pT4) (89.04%), frequently with lymph node metastases (pN+) (58.9%) and with lymphovascular invasion (LVI+) (47.95%).

As related to the analysis of the main clinical and morphological parameters for the lot of 1612 cases of ADK NOS, we noticed that most of the ADK NOS were well differentiated tumors (G1-G2) (86.04%), but mainly diagnosed (85.11%), in advanced stages of the tumor invasion into the intestinal wall (pT3-pT4). Lymphovascular invasion (LVI+) and lymph node

metastases (pN+) were were present at 46.71%, and 49.19%, respectively, of the cases, while proves for the presence of distant metastases (pM+) only occurred in 5.51% of the cases. We have identified, at patients under 60, more frequently tumors at the level of the left colon (41.62%), followed by rectal tumors (31.31%). Except for the left colon which got to be the main localization of tumors both in women (40.80%) and in men (43.18%), the second localization in the order of its frequency, in women – right colon (36.79%), in men – the rectum (30.59%). Most cases with early tumor invasion in the intestinal wall (pT1-pT2) - 43.75% were identified at the level of the rectum.

Mucinous adenocarcinoma (191 cases) were diagnosed mainly in patients >60 years old (73.30%), male (55.49%), at the level of the left colon (58.64%). Most of the mucinous tumors showed morphologic features with an adverse impact on the prognostic and evolution of the disease: advanced stage of the intestinal wall invasion (pT3-pT4), pN+, LVI+.

2. CONTRIBUTIONS TO THE ASSESSMENT OF NEW PROGNOSIS FACTORS IN COLORECTAL CARCINOMAS

We have assessed, **in the second chapter of the specific part**, besides the classic prognostic indicators, a series of ***new morphologic parameters in CRC***, some less known and/or accounted for, others characterized more recently (TB and PDCs), but promising for a more accurate stratification of the risk of CRC patients, by using cheap methods, easily applicable in medical research.

We have analyzed in the first chapter of the specific part a cohort of 1885 CRC cases, but seeing the large lot of patients, we were unable to assess some of the histologic parameters with potential prognostic value, which have not been accounted for in the histology reports and analysis of which would have required for a thing the reevaluation of the smears of each and every case.

2.1. Assessment of Additional Morphological Parameters in CRC

We have interpreted, from the perspective of assessing additional morphological parameters, on a more limited lot of patients, a series of additional classic parameters (TNM AJCC stage of the disease, ulceration and tumor necrosis, PNI) and others described more recently (TILs, the configuration of the tumor invasion front). We have carried out a retrospective study on a lot of 71 CRC cases, out of which 50 consecutive CRC cases, diagnosed and operated upon by classic surgery in 2014 and 21 CRC cases which underwent robot-assisted surgery (the Vinci Xi® Surgical System), in Clinic II Surgery of SCJUPBT, during the period 07/2015 - 07/2016. We have included in the study cases diagnosed by resection parts, and excluded CRC treated by chemotherapy and/or RT prior to surgeries. We also need to mention that since 2015, the SCJUPBT has carried out complex surgeries, robot-assisted, mainly referred to the oncologic pathology of the colon and rectum, facilities which allowed the surgeries, often minimum invasive, with technical control and high precision. The tissue fragments were processed in the Department of Pathological Anatomy (SAP) of SCJUPBT, endowed with modern, performant, apparatus which contributes to the accuracy of the histopathological diagnosis.

Conclusions. Regarding the *configuration of the tumor invasion front*, we noticed that most of the cases (63%) presented a model of infiltrative tumor invasion front. The infiltrative invasion front was considered related to negative impact parameters on the disease evolution like the localization of the tumors at the level of the right colon, the increased degree of malignity (G3-G4), the profound invasion in the intestinal wall (pT3-pT4), advanced stage TNM AJCC of the disease, pN+, pM+, LVI+ și PNI+. Due to its value and ease in assessment, we propose the reporting of this parameter in the histopathologic report. We have obtained results with statistical significance related to the associations between the advanced stage TNM AJCC and the other adverse course parameters (OMS G3-G4 degree, parameters like pT3-pT4, pN+, pM+, LVI+, PNI+, infiltrative configuration of

the invasion front). I have noticed important associations of the PNI to the remaining parameters with a negative role on the prognostic of the CRC patients. As well, the presence of the tumor necrosis was one of the unfavorable prognostic indicators for the CRC patients. In return, the tumor ulceration did not present statistical significance in the carried out multivariable tests. TILs did not correlate significantly statistically to the other investigated parameters, but the assessment method chosen by us may not be the best. The TILs assessment represents a future research direction, but to establish a standardized methodology and generally accepted by the quantification and of additional studies to validate the prognostic significance of TILs in CRC is a need.

2.2. Contributions to the Assessment of *Tumor Budding* (TB) in CRC

As the assessment method of the TB are quite diverse, my own contribution consisted of an attempt to implement and validate a ***simple assessment method of the TB***, which shall be easily reproductible in daily practice and with a limited inter-observer variability. Labelled as TB are solid groups of 1-4 tumor cells which do not represent glandular fragmentation areas caused by the inflammatory infiltrate (25)(29). At the *International Tumor Budding Consensus Conference (ITBCC)*, in 2016, TB was recommended to be included in the histopathology report (30)(31) and the need to enter such parameter in the classification of TNM in case of CRC (32)(33) has also been supported.

I have assessed in the *first part of this study,* TB on scanned slides, HE colored, IHC colored respectively, from the perspective of checking the reproducibility and variability inter-observer: ***pilot study in Timisoara (Romania)***. An original thing about this work is represented by the ***use of digital pathology in CRC assessment***. I have assessed TB on scanned slides come from 21 CRC cases previously diagnosed by endometrial biopsy and undergoing robot surgery (da Vinci Xi® Surgical System) between 07/2015-07/2016, in the Surgery Clinic II of SCJUPBT. I have excluded the patients which underwent neoadjuvant treatment.

The first step in the assessment of the TB consisted of selecting, for each and every case, by evaluation to the optic microscope, at low magnification (objective 10X), of the slide with the tumor section which contained the largest number of TB at the invasion front of the tumor. We have carried out for the IHC determinations additional sections made of paraffin blocks, thickness 3 μm, which were placed on pre-treated slides with silane, in order to prevent their detachment during the pre-treatment procedures used for antigen retrieval. IHC determinations were carried out on the staining automate Leica Bond Max found in the labs of the Morphopathology Department within the UMFVBT, by using anti-CK AE1/AE3 antibodies (Novocastra, ready-to-use) and polymeric detection system Novolink. The visualization was possible by using 3.3'-Diaminobenzidină (DAB), and the nuclei counterstaining by Hematoxylin. In the end, the coverslips were mounted on slides with Entellan.

The TB assessment was carried out on ***virtual slides*** according to the protocol below.

The 21 HE stained slides and the corresponding slides marked IHC (CK AE1/AE3) have been analyzed by three assessors after having been scanned by the slides scanner Leica Aperio AT2, upon magnification 40x. In order for the slides to be anonymous and not to allow the assessors to check the case the slide belongs to, each and every digital slide was assigned an implicit consecutive number, according to the scanner settings. There has never existed a direct labelling to link the digital slides HE to their corresponding IHC slides.

The digital slides resulted were afterwards saved on a local server, by using Leica Digital Image Hub Solo v4, a program which shall allow remote access, by web interface, to all the digital images available on the server. That way, the assessment of the slides was possible from any location with an access to the internet, without the need to install a visualization soft on the assessor's computer. Individual access accounts were created on the server for the three assessors. The set of slides (21 HE and the corresponding 21 IHC) to be assessed was replicated, each of the evaluators only had access to his or her own set of scanned slides, and therefore assessment independence was assured, and the assessors were impossible to be influenced.

At the end of the assessment, the results were gathered in a unique database and the connection between the number of the digital slide and the number of the case, restored.

10 circles were drawn on the scanned sides, along the tumor invasion front, which represented the areas for the quantification of the TB. Each and every circle had the diameter of 0.785 mm^2, in order to simulate the area covered by an objective of the microscope 20x, with the observance of the recommendations for assessment from the literature (31)(34)(35). After a first inspection of the areas assigned by circling, without actually counting the TB, each and every evaluator set which of the 10 drawn circles represented the area with the largest TB density, area which was assigned as *arie hotspot*. The Hotspot was the first field for the assessment, and the procedure was afterwards followed by the quantification of the other fields. After the centralization of the values obtained, the arithmetic mean was calculated for the assessed TB in the ten fields, for each and every case, both on the HE stained slides and on the IHC stained slides.

The classification of the cases in relation to the mean of the TB number was carried out according to a system with 3 TB degrees (GBd): tumors ≤5 TB were classified as G1Bd, those with 6–9 TB as G2Bd, while tumors ≥10 TB were classified as G3Bd, according to the model recommended by Cho and collab. (36). Subsequently, we have classified the tumors according to a system with two TB degrees: low GBd, for tumors ≤9TB and high GBd, for tumors ≥10TB, according to the model used by Graham and collab. (37).

In order to determine the variability inter-observer in the assessment of the TB, we have calculated the intraclass correlation coefficient – ICC and the concordance coefficient (Cohen's Kappa), using the statistics program IBM Statistical Package for the Social Sciences (SPSS) v. 20.

I have analyzed in the *second part of the study dedicated to assessing TB* the extended lot of 71 CRC cases for which I had previously analyzed the significance of the other clinical and morphological parameters. Based on the consideration that according to the WHO classification of the digestive system tumors in 2010, mucinous ADK is not graded depending on the percentage of the glandular structure shaping but on the absence of a consensus related to the definition of TB or PDCs in the presence of the mucin, I have not graded the two cases of mucinous ADK by none of the three grading systems (WHO degree, GBd or PDCs-G). Therefore, the TB quantification was carried out in this second part of my study only for the 69 ADK NOS cases, based on the previously mentioned considerations.

I have carried out the ADK NOS grading on HE stained slides, at the optic microscope, and quantified the presence of TB at the tumor invasion front, according to the recommendations proposed at ITBCC in 2016 (30). After the classification of tumors depending on GBd, we have analyzed the comparison between the new grading system GBd based on TB quantification and the old grading system – WHO degree. And then, we analyzed the correlations between GBd (three degree class GBd, two degree classes respectively) and all the other investigated parameters in order to check if the classification system based on the TB quantification is a promising prognostic parameter in assessing the CRC.

Conclusions. From the perspective of TB assessment in CRC, in order to standardize the assessment method, the inter-observer variability appreciation and the analysis of the relations to the other investigated parameters, I have drawn important conclusions. We consider the TB assessment method on 10 microscopic fields as feasible and reproductible method. It eases the teaching and training of pathologists in order to quantify TB and offers a good inter-observer agreement in TB assessment. The number of TB was higher than on the IHC stained slides and was associated to a better inter-observer agreement, underlining the utility of IHC for the accuracy of TB assessment. One of the advantages to use IHC and was also the ease in identifying TB by unexperienced pathologists. I have also noticed a better agreement amongst observers for the classification of tumors based on TB assessment in the GBd system with two-degree classes (binary), as compared to the GBd system with three levels. The high degree TB is a marker associated to adverse prognostic indicators. Both with the aid of the binary system and with that with three classes, I have reached significant correlations between the TB degree and the other investigated parameters, but

due to the ease in the assessment and to a low inter-observer variability, the binary grading system of the TB seems to be more useful to classify CRC cases.

By using the assessment method based on TB quantification in the hotspot area, recommended by the ITBCC, I have noticed results similar to those obtained by using the grading method based on the average value of the quantified TB in 10 microscopic fields. The advantage of the "hotspot" method to assess TB consists of the quickness of the pathologist to assess TB and implicitly in daily practice applicability. Out of the comparative analysis of the GBd grading system to the usual grading system – WHO degree, I have noticed more significant correlations of the GBd to some of the investigated parameters. Therefore, the GBd high degree was associated to pN+, the advanced stage TNM AJCC of the disease and to LVI+, and therefore, in all the situations, we reached to ***p*** values of 0.0001. Multivariable analysis showed significant correlations of the GBd to other parameters as well: the tumor invasion front – infiltrative tyoe (p=0.0002), PNI+ (0.0022), pM+ parameter (p=0.034) and the presence of tumor necrosis (p=0.0347).

2.3. Contributions to the Assessment of *Poorly Differentiated Clusters* (PDCs) in CRC

There is a significant inter-observer variability in grading CRC, due to the use of several classification systems and to the absence of explicit criteria to frame them in a certain degree category (24)(38). WHO grading of CRC is based on the quantification of making glandular structures (percentual method) (24)(38). Although this system is used on a large scale, it is encumbered by subjectivism, as glands shaping is difficult to estimate (39). Additionally, there are controversies related to the area to be assessed – the less differentiated area (increased degree) or main degree (40). As well, for the signet ring cell carcinoma or micropapillary carcinoma, the degree of the tumor, set according to such criteria, it has uncertain prognostic value (39)(40). Even more, according to the WHO classification in 2010 of the digestive system tumors, the assessment based on the glandular differentiation of CRC is only applied for conventional / classic / ADK /NOS, except for special versions – mucinous ADK, signet ring cell carcinoma and medullary carcinoma (41). Therefore, the utility of this system is questionable.

I set out to assessed, within my ***study dedicated the assessment of PDCs***, a new histologic grading system of the CRC - PDCs (PDCs-G) degree, based on the quantification of the groups of poorly differentiated clusters (PDCs). I have analyzed the clinicopathological and prognostic significance of the PDCs-G, as opposed to the usual grading system (WHO degree). The study material was represented by the lot of 71 CRC cases, which had been previously been subject of my analysis related to the correlations amongst additional morphologic parameters, but, seeing that there is no clear definition of the clusters in mucinous areas, I have not interpreted the two cases of mucinous ADK, the PDCs quantification was carried out as such only for the 69 cases of ADK NOS.

PDCs are defined as solid groups/clusters of ≥5 tumor cells, without the formation of glands (24). I have realized that ADN NOS grading depending on the presence of PDCs at the tumor invasion front, on HE stained slides, according to the method entered by Ueno and collab. In 2012 (24). To this effect I have selected for each and every case, the proper block for the tumor section which included the invasion front, from the area with maximum tumor infiltration in the intestinal wall.

All the slides selected (one sample /case) were scanned in a first step by the slide scanner Leica Aperio AT2. The slides were initially examined at little magnification (x40), in order to identify the area with the largest density of PDCs along the invasion front. This area was considered Țhotspot" and subsequently assessed at intermediary magnification (200x), for which the field dimension was 0.785 mm^2. Depending on the number of PDCs identified in the analyzed area, I have classified the CRC cases in 3 categories of PDCs degree: tumors <5 PDGs were considered PDCs-G1, tumors with 5-9 PDCs were classified as PDCs-G2 category, and the cases with ≥10 PDCs were considered PDCs-G3 (42).

I have subsequently compared the two grading systems of CRC (WHO degree and PDCs-G) and have analyzed their relation to the other clinicopathologic parameters, in order to see if the new classification system based on PDCs quantification is an additional histologic instrument in assessing the CRC that is a new promising prognostic indicator for CRC patients.

Conclusions. I have noticed that the highest degree PDCs-G correlates to the clinico-morphological parameters related to the unfavorable course of CRC, especially to great deepness of the tumor invasion (pT3-pT4), the advanced TNM AJCC of the disease, the lymph nodal metastases (pN+), the invasion of lymphatic vessels (LVI+), perineural invasion (PNI+) and the infiltrating configuration of the tumor invasion front, with more statistically significant values than those obtained by the classification of the cases with the WHO grading system. Additionally, our data show that the new classification system of CRC, based on PDCs quantification (PDCs-G), represent an independent predictor for the presence of the lymphovascular invasion and of the lymph node metastases. Both TB and PDCs are correlated to the same unfavorable prognostic indicators, but seeing that the PDCs are easily to assessed as opposed to the TB, on ordinarily stained slides, the PDCs seems to be a more reproductible and valuable parameter than the TB.

MY OWN CONTRIBUTIONS

The value of this doctoral study stays in the great number of CRC cases, analyzed in the first part of my research. I have drafted a retrospective study, extended over a period of 10 years (2009-2018), where I carried out a ***large database*** which included the parameters selected from the histopathologic reports and from the cover sheets of the biopsy material from a group of ***1885 CRC cases***. This database, extremely comprehensive, will contribute, if continued, to fulfil the Regional Cancer Registry for the western part of Romania, and implicitly of the National Cancer Registry, in order to align to the standards and recommendations of IARC WHO (The International Agency for Research on Cancer of the World Health Organization). Based on the data collected, we can shape a profile of the patient /colorectal tumor, to offer useful information in order to draft a regional or national program to control cancer and in order to implement the screening programs for CRC. Even more, I have assessed many clinico-morphological parameters, some classic, other newer, inconstantly accounted for or not yet included in the guides and protocols dedicated to assess the CRC. Therefore, this database can serve as the departing point to layout subsequent directions and studies for the research of CRC.

The originality of my work consists of analyzing CRC cases undergoing robotic surgery and in assessing TB on scanned slides. Due to the modern apparatus held by the surgical departments within SCJUPBT, I had the opportunity to study a group of 21 ***CRC cases undergoing robotic surgery.*** As well, by processing the tissue fragments in the SAP laboratory within SCJUPBT which is endowed with high-end equipment, I have benefitted from the analysis of the CRC cases on HE and IHC stained slides at excellent quality standards. Using the slide scanner Leica Aperio AT2, held by the Morphopathology Department of the UMFVBT, I was able to analyze a series of parameters on scanned slides. Therefore, an original aspect of my research is represented by the ***use of digital pathology*** which, according to our knowledge, was less used in the study of CRC in Romania. Seeing the current background, of COVID-19 pandemic, I had the opportunity to take over photos of interest from scanned slides to my home. Additionally, by using virtual microscopy, I was able to reduce interobserver variability and to standardize the assessment and quantification of the investigated parameters.

As TB represents a promising prognostic indicator in CRC, but the assessment methods are very diverse, one of my **own contributions** was an attempt to ***implement and validate a simple assessment method of the TB***, easily reproductible in daily practice with a low interobserver variability. Due to such perspective, I have tried, for the first time in

Romania, according to our science, to put together a TB quantification method. I have selected a group of CRC cases undergoing robotic surgery for which I chose to assess TB, in ten microscopic fields, according to the recommendation in literature, from the moment this study was started. This method enables the training of pathologists in quantifying TB and offers a great interobserver agreement and proved to be a feasible and reproductible method. The assessment method of the TB on 10 microscopic fields can represent a first step for standardizing the TB assessment in pathology labs where one can scan the slides. Additionally, according to the ITBCC recommendations in 2016, I have chosen to quantify TB, in order to validate the prognostic significance of this parameter, on a larger lot of patients, but with assessing to the optic microscope one single area (hotspot), at the tumor invasion front of the tumor, one of the advantages being the ease of the assessment. Due to the applicability and reduction of the interobserver variability, proved by our results, the binary GBd system would rather or recommended to be used in CRC assessment. We suggest for this grading system for the TB by two-degree classes (with setting a cutt-off of 10 TB), to be implemented for the TB routine accounting for, with mentioning the degree category (GBd) and the specific number of TB in the pathologic report.

A different **contribution of my own** is represented by the ***assessment of the PDCs for the first time in Romania*****,** according to my knowledge. Globally as well, there are few studies which compared directly the PDCs with conventional histologic degree of the tumor, but there are proofs that the PDCs-G make a more uniform distribution of the CRC cases amongst the three degree classes and it would be a more robust prognostic indicator than the WHO degree in CRC.

Seeing the relationship between the PDCs and TB (already included in several international guides for CRC), we plead for the inclusion in the histopathology report both of the TB and the PDCs. If our results are confirmed by other researches as well, TB and PDCs should be included amongst the prognostic indicators from TNM classification. Additionally, digital pathology may be applied in order to improve standardization and the precision to assess TB and PDCs.

Consequently, TB and PDCs serve as parameters to be known, and the pathologist needs to be familiarized with their identification, quantification and reporting methods, as standardizing such assessments will lead to the implementation of these new prognostic and predictive indicators in the current diagnosis of the CRC. As well, TB and PDCs should be taken into account within multidisciplinary meetings in order to assist therapeutic decision making in daily clinical practice.

The topic I've chosen is scientifically contemporary, to improve the therapeutic management of CRC being the center of national and international scientific preoccupations.

A part of my doctoral study materialized by the publication of two original articles in medical magazines and the presentation and publication of summaries at international scientific conferences.

By the topic I have approached, this paper fits into the research directions of UMFVBT and into the themes of interest of the research team within the Morphopathology Department within UMFVBT, where I've carried out my work of research for this doctoral study.

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