MEDICAL UNIVERSITY
"PROF. DR PARASKEV STOYANOV" – VARNA

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PREDICTIVE AND PROGNOSTIC ROLE OF MARKER FOR NECROPTOSIS – RIPK3 IN PATIENTS WITH COLON CANCER IN METASTATIC STAGE

DISSERTATION SUMMARY

FOR THE ACQUISITION OF THE EDUCATIONAL AND SCIENTIFIC DEGREE

"DOCTOR (PhD)"

SCIENTIFIC SUPERVISOR:

ASSOC. PROF. ELEONORA GEORGIEVA DIMITOVA-GOSPODINOVA, M.D. PhD

VARNA, 2022

The dissertation consists of 100 pages and is is illustrated with 15 tables and 19 figures. The bibliographic references include 227 sources, of which 227 are in Latin.

The dissertation was discussed and approved for defence in a department meeting of Department of Oncology, Faculty of Medicine, Medical University of Varna and with an order of the Rector of MU-Varna the following scientific jury was appointed:

External members:

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The public thesis defence will be held on 26.05.2022 in a virtual meeting in the online Blackboard platform of Medical university of Varna.

Thesis materials are available in the Library of Medical University – Varna and online on the University's website (mu-varna.bg).

Contents:

1. Abbreviations

5-FU – 5-fluorouracil

AJCC - American Joint Committee on Cancer

CEA - carcinoembryonic antigen

ECOG - Eastern Cooperative Oncology Group

KRAS - Kirsten rat sarcoma viral oncogene homolog

MLKL - Mixed Lineage Kinase Domain Like Pseudokinase

PET/CT - Positron emission tomography–computed tomography

RIPK1 – receptor-interacting protein kinase 1

RIPK3 - receptor-interacting protein kinase 3

CRC – colorectal cancer

CT – computer tomography

CBC – complete blood count

2. Introduction

Colorectal cancer (CRC) is the third most common tumor worldwide and one of the leading causes of death from cancer in both sexes. While surgical treatment remains the main therapeutic approach in patients with early-stage CRC (I-II), chemotherapy is the option of choice for metastatic or unresectable disease (stage IV).

About 2/3 of patients with CRC develop distant metastases, with the liver being the most common metastatic focus. About 15-25% of patients with CRC have metastatic lesions at the time of diagnosis, while another 18-25% of them develop distant metastases within 5 years after the initial diagnosis. 5-fluorouracil (5-FU), a synthetic pyrimidine analogue, is the standard chemotherapeutic drug used for advanced CRC and, in combination with other agents, achieves initial response levels of 40-50%.

Over the last 50 years, despite the many benefits of 5-FU, its clinical applications have been severely limited due to the available drug resistance. With the advent of effective systemic chemotherapy combined with biological agents, the discovery of new prognostic and predictive factors that help better understand the biological behavior of the tumor, predict the effect of chemotherapy, and earlier diagnosis, there is a significant improvement in 5-year survival in patients with advanced metastatic disease.

Cell death by apoptosis is a natural barrier to the development of cancer, as it limits the uncontrolled proliferation induced by oncogenes. In recent years, a growing number of studies have shown that there are other genetically programmed types of cell death other than apoptosis. This type is necroptosis - a process strictly regulated by different molecules and combining morphological characteristics of both necrosis and inflammation.

Necroptosis is a newly discovered pathway of regulated apoptosis, the action of which requires the involvement of receptor-interacting

protein kinases 1 and 3 (RIPK1 and RIPK3), as well as the mixed lineage kinase domain-like protein (MLKL). As necroptosis is increasingly considered an important process in the pathogenesis of cancer, a deeper understanding of the mechanisms of necroptosis is essential for the development of new approaches to its regulation in neoplastic processes.

Resistance to apoptosis is a major factor in the failure of chemotherapy during treatment. Bypassing the apoptotic pathway to induce cancer cell death is considered a promising approach to overcoming this problem. Necroptosis is a regulated necrotic cell death in a caspase-independent manner and serves as an alternative to programmed cell death, overcoming resistance to apoptosis by enhancing antitumor immunity in cancer treatment.

Resistance to 5-FU-based chemotherapy is a key challenge in the treatment of patients with mCRC and new targeted approaches are needed to improve therapeutic outcomes. Therefore, the discovery of new predictive and prognostic markers is essential to improve treatment outcomes in patients with metastatic colon cancer.

3. Aim and objectives of the dissertation

3.1 Aim of the study

The aim of the study is to look for a correlation between the expression of RIPK3 in the primary tumor in patients with metastatic colon cancer and some clinical and pathological characteristics - sex, age, degree of tumor differentiation, KRAS mutation status, its relationship to biological behavior of the tumor, as well as progression-free survival and overall survival.

3.2. Objectives

1. Selection of patients with colon cancer in metastatic stage.

2. To compare the levels of immunohistochemical expression of RIPK3 in the primary tumor of patients with colon cancer.

3. To analyze the correlations between the immunohistochemical levels of RIPK3 expression with the clinical and pathological characteristics of patients with colon cancer.

4. To analyze the predictive value of RIPK3 expression in the primary tumor to respond to 5-FU based first-line chemotherapy.

5. To analyze the prognostic potential of RIPK3 expression in the primary tumor in terms of progression-free survival and overall survival.

4. Materials and methods

4.1. Center of research

1. Clinic of Medical Oncology at the University Hospital "St. Marina" - Varna

2. Laboratory of Clinical Immunology at the Department of Medical Genetics

3. Medical University and University Hospital "St. Marina" – Varna

4.2. Patient population

We conducted a retrospective non-interventional single-center clinical trial, which examined the cases of 74 patients with unresectable, metastatic colon cancer. The study included patients treated with first-line 5-FU-based chemotherapy from January 2012 to December 2015 at our University Hospital "St. Marina". They were all staged with CT or PET/CT before starting treatment. Some patients received adjuvant chemotherapy. Performance/ECOG status of patients was assessed at <2 *(Table 1).* All of them met the inclusion criteria and there are no exclusion criteria.

Including criteria:

1. Patients over 18 years

2. Histologically proven colon cancer

3. Patients in IV clinical stage - unresectable or metastatic CRC

4. Conducted first line chemotherapy with 5-FU based chemotherapy in the University Hospital "St. Marina" - Varna for their metastatic disease

5. ECOG PS - performance status < or = 2

6. Values of some laboratory parameters such as blood count, renal and hepatic function within reference limits

Excluding criteria:

1. Patients with impaired general condition - ECOG PS over 2 according to AJCC

2. Radically operated patients without secondary lesions

3. Severe and uncontrolled concomitant diseases

4. Serious deviations from laboratory parameters

4.3. Medical history of the patients

A separate medical record was created for each patient in the trial, including the following information, where available:

Demographics

Names (Initials):

ID number / date of birth:

Age:

Gender: male / female

Medical history

Performance status / ECOG: The general condition of each patient according to ECOG was initially assessed as 0, 1 or 2

Concomitant diseases:

Cancer information

Clinical diagnosis: localization of the primary tumor, TNM staging, degree of differentiation

Date of operation / if any /

Histological result: number, date, number of paraffin blocks

Localization of the primary tumor:

Localization of distant metastases:

Molecular pathological analysis of RAS:

Date of initiation of systemic first-line chemotherapy:

Type of systemic chemotherapy

Baseline and control imaging studies: CT, PET / CT, RECIST assessment 1.1

Progression free survival

Overall survival

Table 1. Assessment of the performance status /PS/ according to the Eastern Cooperative Oncology Group /ECOG/ scale.

Stage	ECOG /PS/ status
0	**Fully active, able to carry on all pre-disease performance without restriction**
1	**There are restrictions on physical activity, but the patient is able to perform light, non-strenuous or sedentary work.**
2	**The patient is capable of take care of himself, but is unable to work. He spends> 50% of the time he is awake upright**
3	**The patient is only capable of limited self-care. He spends> 50% of the time he is awake in bed or in a chair**

4	**The patient is completely unable to take care of himself, completely confined to the bed or chair**
5	**Dead**

4.4. Routine clinical trials and biological markers

The necessary parameters were defined in advance and monitored retrospectively during the study.

1. Haematological, biochemical results and CEA values from routine tests.

2. Imaging - baseline CT or PET/CT, as regular ones during systemic therapy to evaluate treatment.

3. Tumor block from the primary tumor for histological examination: fixation in neutral formalin, inclusion in paraffin blocks, standard staining with hematoxylin-eosin and staining to assess RIPK3 tumor expression.

4.5. Specific test methods

Preparation of biopsy materials for immunohistochemical examination

Samples of primary adenocarcinoma of the colon from 74 patients were examined in the Department of Clinical Pathology of the University Hospital "St. Marina". Five micrometer sections were cut from the paraffin blocks and placed on slides. The sections were dewaxed with xylene and dehydrated in a graduated series of ethanol. Antigen recovery was performed in preheated EnVision FLEX Target Retrieval Solution (working solution) in PT Link tanks and incubated for 30 minutes at 97 ° C with pH = 9. After cooling, the slides were placed in diluted FLEX wash buffer at room temperature (20x) for 1-5 minutes. The samples were tested with recombinant rabbit

polyclonal antibody against RIPK3 (ABCAM's RabMab technology ab56164). The antibody (Anti-RIPK3, diluted 1: 100) was incubated for 20 minutes. Detection of RIPK3 expression levels was achieved using the UltraVision Anti-polyvalent detection system, HRP / DAB. Finally, the reaction was visualized with a substrate-chromogen (DAB, diaminobenzidine) reagent. Counterstaining was performed with Mayer's hematoxylin to assess immunostaining, and digital images were obtained using a Leica Aperio ScanScope AT2 (Aperio Technologies, Vista, CA).

Determination of RIPK3 expression levels and interpretation of results

Immunohistochemical expression of RIPK3 was assessed using the H-score (histo-score) on tissue samples. For each cell in different fields determine the membrane intensity - 0, 1+, 2+, or 3+. The percentage of positive cells for each intensity was calculated, and finally the H-score was calculated using the following formula: [1x (% cells with 1+) + 2x (% cells with 2+) + 3x (% cells with 3+)] , ranging from 0 to 300. The H-score was also used to assess RIPK3 nuclear expression. The result was obtained by the formula: [1x (% of weakly positive nuclei1 +) + 2x (% moderately colored nuclei 2+) + 3x (% of strongly positive nuclei3 +)], in the range 0 to 300. The average value was used to estimate the RIP3 expression and grouping patients into two categories of low and high expression *(Figure 1).*

Determination of RAS mutation status

To study the RAS mutation status of the test samples, 5 μm thick tissue samples from the paraffin blocks that were subjected to dewaxing. DNA was extracted from formalin-embedded paraffin-fixed tissue, and then the isolated DNA was examined for mutations in exons 2, 3 and 4 of the KRAS gene using an allele-specific real-time quantitative PCR-based analysis apparatus. qPCR System (7500 ABI PRISM, Applied Biosystems, USA) at the National Genetic Laboratory - Sofia.

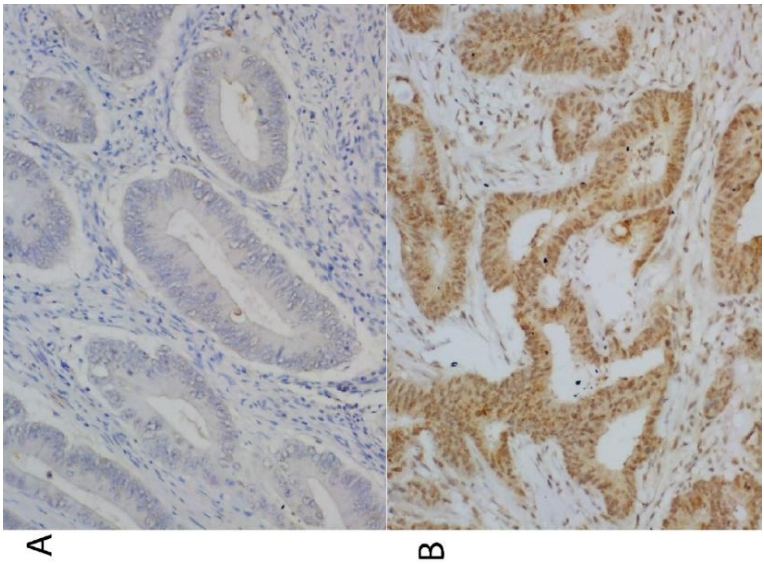

Figure 1. Immunohistochemical staining of RIPK3 in human colon carcinomas (20x magnification) A - a patient with low RIPK3 expression. B - patient with high expression of RIPK3.

4.6. Type and duration of systemic drug therapy

All patients received at least three months treatment with 5-FU based first-line chemotherapy. The majority of patients with 55.4% (n = 41) were treated with FOLFOX4 / CAPEOX ± Bevacizumab / Panitumumab. 29.7% of patients (n = 22) underwent FOLFIRI ± Bevacizumab / Panitumumab treatment, and the remaining 14.9% (n = 11) used the De Gramont regimen *(Figure 2).*

The regimens were repeated every two weeks until disease progression was recorded, with each patient receiving treatment for at least 3 months. A total of 60 patients (81.1%) received the full dose of the chemotherapeutic drugs used, while 18.9% (n = 14) had to adjust the dose by reducing or skipping the cycle due to reported toxicity. CBR was reported in 35.1% of patients. Patients with low RIPK3 expression showed significantly lower levels of response to treatment

than those with low expression (16.2% vs. 54.1%). In addition, given the CBR response to chemotherapy, high RIPK3 expression is a positive prognostic marker for the response rate (OR 0.165, 95% CI 0.05-0.49, P = 0.01).

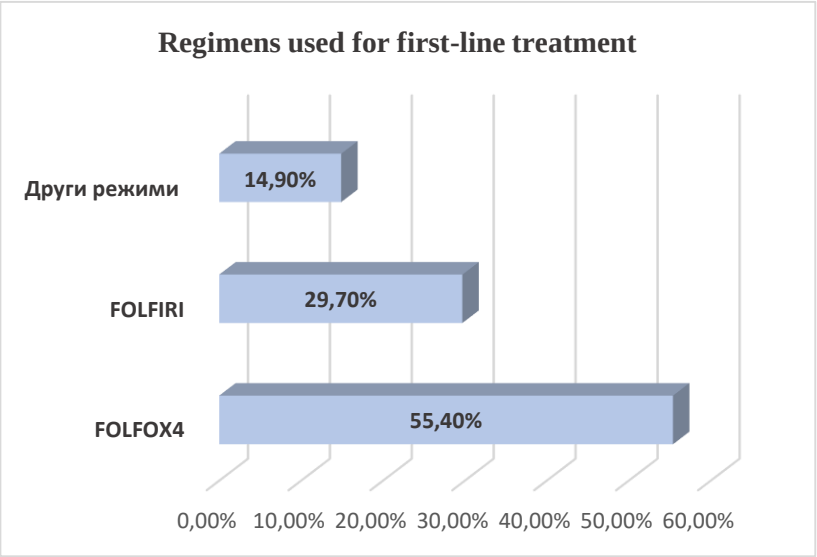

Figure 2. Distribution of the regimens used for first-line systemic chemotherapy.

4.7. Imaging assessment of the therapeutic response

Imaging in all patients was performed before the start of first-line treatment, followed by regular every three months (4-6 chemotherapy cycles) during systemic treatment until disease progression was recorded according to RECIST 1.1 criteria. The imaging methods used are CT of the chest, abdomen and pelvis or by PET / CT. In the course of the systemic treatment, the parameters PFS (progression-free survival), OS (overall survival) were monitored, where it can be determined. The types of response to the treatment

according to the RECIST criteria 1.1. and according to the adapted EORTC criteria are shown in Table. The types of answers are:

- CR (complete response) - disappearance of all target lesions

- PR (partial response) - reduction by 30% or less of the sum of the longest diameters of the target lesions compared to the initial measurement

- SD (stable disease) - lack of sufficient reduction in the size of the lesions, as well as a sufficient increase to assess the progression of the disease, comparing the smallest sum of the longest diameters against the background of treatment.

- PD (progression of the disease) - the appearance of one or more new lesions or at least a 20% increase in the amount of the longest diameters of the target lesions relative to the smallest amount compared to the study (baseline or control during treatment).

- Clinical benefit rate (CBR) - is defined as the percentage of patients with advanced or metastatic cancer who have achieved a complete response (CR), partial response (PR) and stable disease (SD) in response to therapeutic intervention in anticancer clinical trials.

4.8. Statistical design and analysis

The statistics were processed using the software package SPSS version 23. The values are presented with their mean values ± standard deviation. Differences with $p \leq 0.05$ were considered statistically significant. The following statistical methods were used in data processing:

- Descriptive analysis of statistical values - mean, standard error of the mean, minimum and maximum values, median.

- Graphic methods - for distribution of patients into groups are used pie and pie-pie charts, linear graphics.

- ***Correlation analysis*** - coefficients of linear correlation according to Spearman (parametric method) are determined. The following correlation strength scale is used to interpret the results of the correlation analysis according to the rho value:

- **< 0.19** – very low correlation
- **0.19 ÷ 0.39** – low correlation
- **0.40 ÷ 0.59** – median correlation
- **0.60 ÷ 0.79** – high correlation
- **≥ 0.80** – very high correlation

- ***The relationship between OS and PFS and the level of RIPK3 expression in the primary tumor was investigated*** - the time to event and the endpoints were analyzed by the Kaplan-Meier method, and the differences were assessed using a log-rank test

- ***Mann – Whitney U test and χ2 test were used*** - to compare and evaluate the correlations between the expression of RIPK3 in primary tumors and clinical and pathological characteristics of patients such as age, sex, KRAS status, histopathological degree

5. Results

5.1. Clinical and pathological characteristics of patients

We conducted a retrospective study, which included 74 patients with histologically proven colon cancer in the metastatic stage, conducted first-line systemic chemotherapy at the University Hospital "St. Marina" - Varna. The percentage distribution of patients by groups according to the different clinical and pathological parameters is presented in *Table 2*.

Table 2. Distribution of the total number of patients by groups according to some demographic, clinical and pathological characteristics.

Sex (%, n)	
Male Female	56.8 % (n=42) 43.2 % (n=32)
Age at the time of diagnose (%, mean ±SD)	
Male	66.09 (±7.734)
Male	64.76 (±8.118)
Summary	63.42 (±10.476)
Tumor localization (%, n)	
Left colon	67.6 % (n=50)
Right colon	32.4 % (n=24)
Metastasis localization (%, n)	
Liver	81.1 (10.0)
Lung	13.5% (28.0)
Peritoneum	12.2% (62.0)
Histological grade (%, n)	
Grade 1	0 % (n=0)
Grade 2	83.8% (n=83.0)
Grade 3	16.2% (n=14.0)

Performance status/ECOG (%, n)	
0	**40.5 % (n=30)**
1	**54.1 (n=40)**
2	**5.4 % (n=4)**
KRAS status (%, n)	
KRAS mutation	**35.1 % (n=25)**
KRAS wild type	**64.9 % (n=48)**

The gender distribution is in favor of men by 56.8% (n = 42), compared to 43.2% (n = 32) for women *(Figure 3).* The average age at diagnosis in men is 66.09 years, and in women 64.76 years. Average age of participants 63.42, with a minimum of 24 years and a maximum of 83 years.

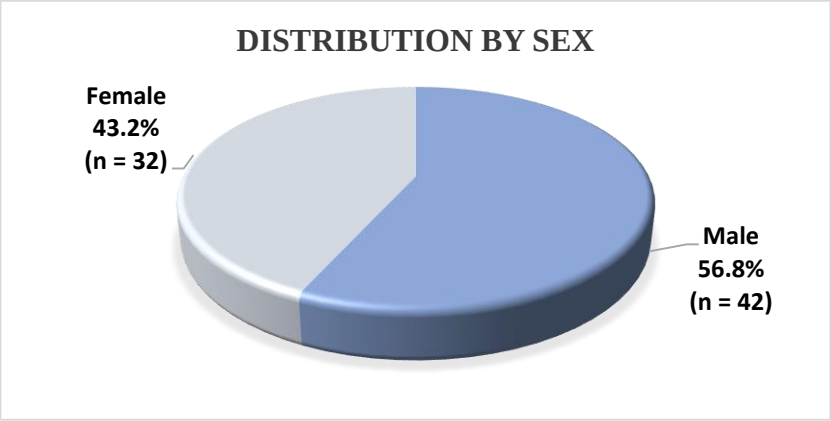

Figure 3. Pie chart depicting patient distribution by sex

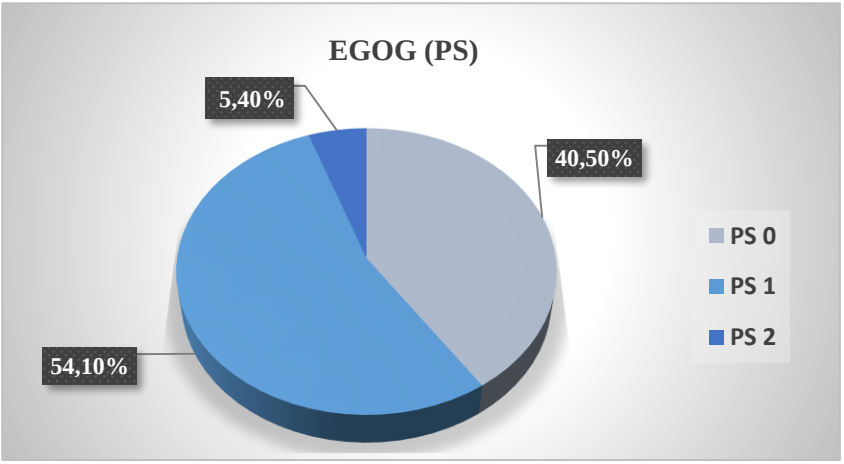

Figure 4. Pie chart depicting patient distribution by ECOG (PS) status.

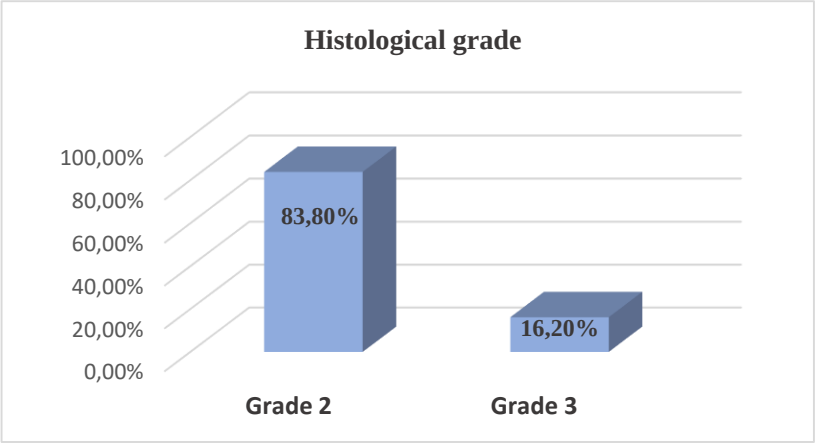

Figure 5. Bar graph depicting patient distribution by histological grade.

Patients with a general condition <or = 2 were included in the study, according to the ECOG scale *(Table 2).* The majority of patients have ECOG PS = 1 - 54.1% (n = 40). 40.5% (n = 30) of them were in very

good general condition with PS = 0, and only four of the general group (5.4%) were with ECOG PS = 2 *(Figure 4).*

KRAS status was studied in all patients. The predominant share of the patient population has a proven KRAS wild-type mutation status - 65% (n = 48), while the remaining 25 patients (35%) have a proven mutation *(Figure 6).*

According to the location of the primary tumor, patients whose neoplasm originates from the left colon predominate by 67.6% (n = 50), while tumor processes from the right colon are only 32.4% (n = 24) of the study population *(Figure 7).*

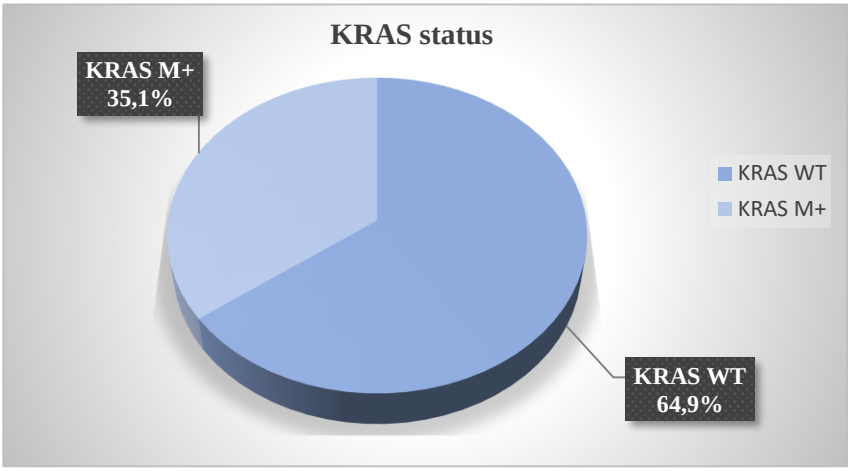

Figure 6. Pie chart depicting patient distribution by KRAS status.

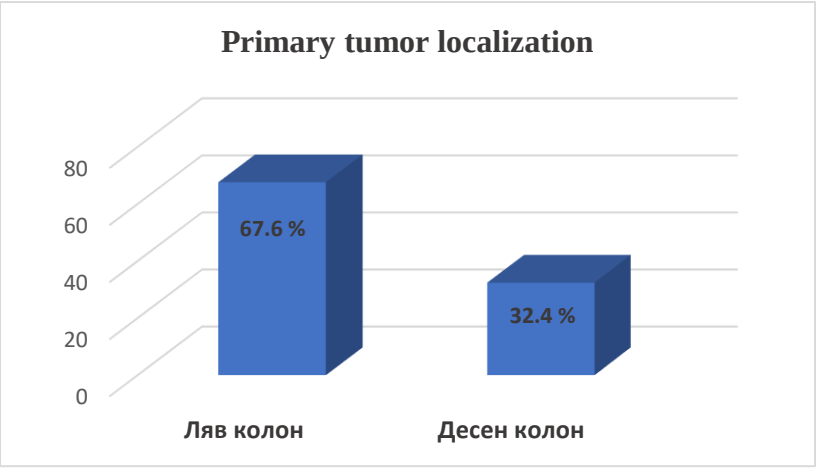

Figure 7. Bar graph depicting patient distribution by the localization of the primary tumor.

5.2. Correlation analys between RIPK3 expression and clinicopathological characteristics of patients

There was no significant correlation between RIPK3 expression and age, sex, KRAS status, and degree of patient differentiation in the study population. CBR (PR + SD for at least 6 months). Patients with low RIPK3 expression showed significantly lower response rates than patients with high RIPK3 expression (16.2% vs. 54.1%) *(Table 3).* In addition, given the CBR response to chemotherapy, high RIPK3 expression can be reported as a positive prognostic marker for the response rate (OR 0.165, 95% CI 0.05-0.49, P = 0, 01).

Table 3. Relationship between RIPK3 expression and some patient indicators. SD - stable disease, PR - partial response, PD - disease progression, M + - KRAS mutation, WT – KRAS wild type

Index	*Low-level RIPK3 expression*	*High-level RIPK3 expression*	*P-value*
Age			
<65	19 (51.4)	18 (48.6)	0.81
≥ 65	18 (48.6)	19 (51.4)	
Sex			
Male	23 (54.8)	19 (45.5)	0.34
Female	14 (43.8)	18 (56.3)	
Histological grade			
G2	28 (45.2)	34 (54.8)	0.07
G1	9 (75.0)	3 (25.0)	
Metastastic site			
Liver			
Yes	29 (45.2)	31 (83.8)	*0.55*
No	8 (21.6)	6 (16.2)	
Peritonium			
Yes	3 (8.1)	6 (16.2)	*0.28*
No	34 (91.9)	31 (83.8)	
Lung			
Yes	7 (18.9)	5 (13.5)	*0.52*
No	30 (81.1)	32 (86.5)	
Chemotherapy response			
SD + PR	*6 (23.1)*	*20 (76.9)*	***0.001***
PD	*31 (64.6)*	*17 (35.4)*	
KRAS			
M+	14 (53.8)	12 (46.2)	0.62
WT	23 (47.9)	25 (52.1)	

5.3. Prognostic value of the degree of differentiation

In the retrospective study, the number of patients with moderately differentiated T2 tumors was predominant (n = 84), while the number

of low-grade carcinomas was only 14. Patients with moderately differentiated tumors showed a significantly longer overall survival of 31.3 (95% Cl, 23.7 - 39.1) months compared to the group of patients with G3 differentiation, in whom OS was 13.8 months (95% Cl, 10.4 - 17.6) (log-rank test p <0.018) *(Figure 8).* No significant association was found between RIPK3 expression and the degree of differentiation *(Table 3).*

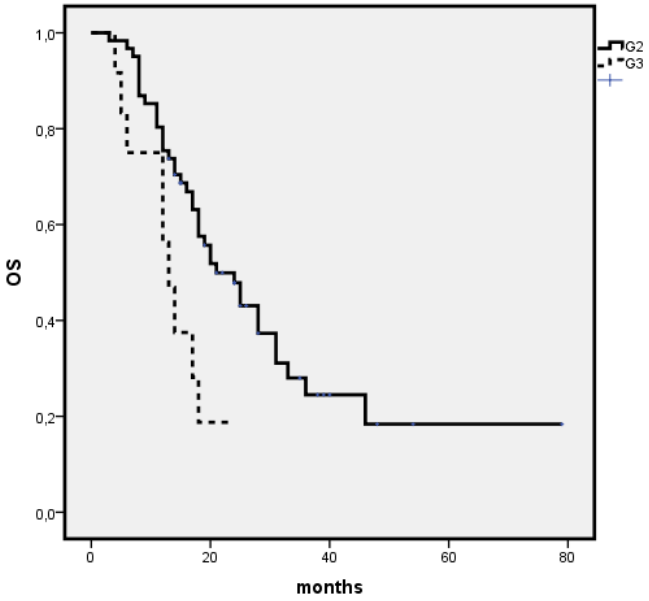

Figure 8. Kaplan-Meier curve of the distribution of total survival according to the degree of differentiation. The mean OS for patients with moderately differentiated G2 tumors was 31.4 (95% Cl, 23.7 - 39.1) months compared to the group of patients with G3 differentiation, for whom OS was 13.8 months (95% Cl, 10.4 - 17.6) (log-rank). test p <0.018)

5.4. Effect of RIPK3 expression on overall survival and progression-free survival

We conducted an immunohistochemical study of the expression of RIPK3 in material from the primary tumor of patients treated at the Clinic of Medical Oncology of the University Hospital "St. Marina" in the period from 01.01.2012. to 31.12.2015 No correlation was found between RIPK3 expression in the primary tumor and gender, the degree of carcinoma differentiation and KRAS status. There was a significant difference in mean progression-free survival (PFS) for the low-expression group compared to the high-expression RIPK3 group (5.6 months) (95% CI, 4.6 - 6.8), versus 8.4 months (95% CI, 6.4 - 10.3) for the high expression group (log rank test p = 0.02) *(Figure 9).*

A significant difference was also reported in terms of overall survival in the two groups. The mean OS for the group of patients with low RIPK3 expression was 18.5 months (95% CI, 15 - 21.9) compared to the group of patients with high expression where PFS was 29.3 months (95% CI, 20.8 - 37.9) (log-rank test p = 0 .036) *(Figure 10).*

Using univariate Cox-proportional regression analysis, it was found that patients with high levels of RIPK3 expression were associated with a lower risk of disease progression HR 0.61 (95% CI, 0.38-0.97; p = 0.044). In the multivariate assay, high RIPK3 expression was an independent factor in predicting longer-term PFS (HR 0.60, 95% CI 0.37-0.98, P = 0.045) *(Table 4).* Also in the one-way assay, high levels of RIPK3 expression were associated with longer OS HR 0.59 (95% CI, 0.35-0.98; p = 0.044). This relationship was not demonstrated in the multivariate HR 0.65 assay (95% CI, 0.38-1.11; p = 0.11*) (Table 5)*. The degree of tumor differentiation was an independent prognostic marker in terms of overall survival (p <0.05).

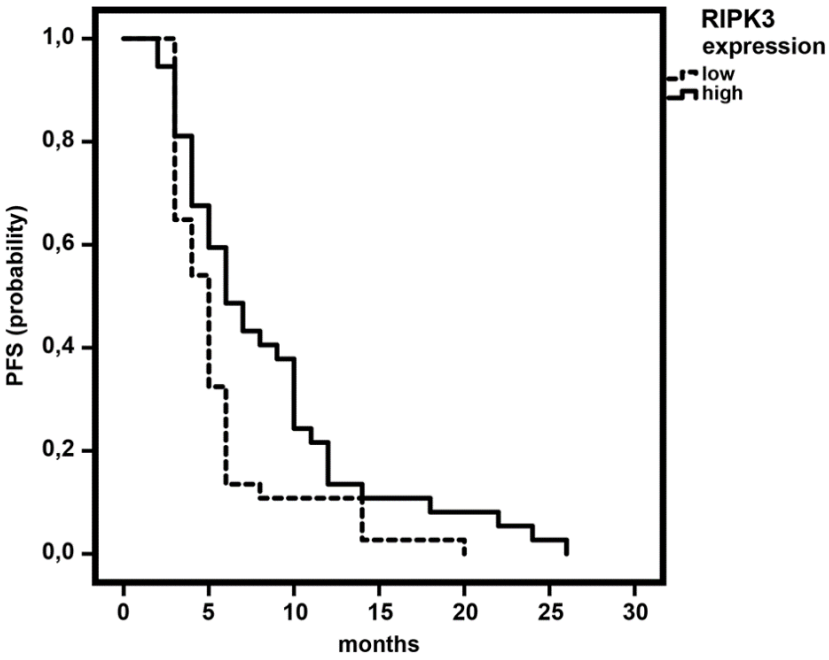

Figure 9. Kaplan-Meier progression-free survival distribution (PFS) curves. The mean PFS for the low RIPK3 expression group was 5.6 months (95% CI, 4.4-6.8) compared to the high expression group where the PFS was 8.4 months (95% CI, 6.4-10). 3) (log-rank test p = 0.02).

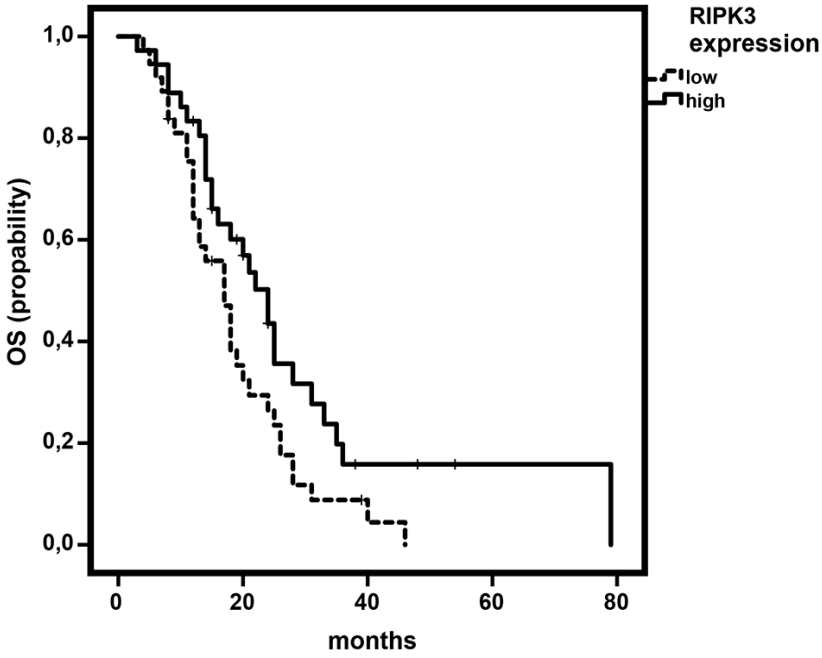

Figure 10. Kaplan-Meier curves of total survival distribution (OS). The mean OS for the group of patients with low RIPK3 expression was 18.5 months (95% CI, 15 - 21.9) compared to the group of patients with high expression where PFS was 29.3 months (95% CI, 20.8 - 37.9) (log-rank test p = 0 .036).

Table 4. Results of Cox regression analysis for predicting progression free survival

Index	Univariate analysis			Multivariate analysis		
Variable	*Hazard ratio*	*95% CI*	*P-value*	*Hazard ratio*	*95% CI*	*P-value*
Age	*1.005*	*0.63-1.59*	*0.1*	*0.91*	*0.56-1.47*	*0.7*
Gender	*0.84*	*0.52-1.34*	*0.4*	*0.95*	*0.58-1.55*	*0.8*
Degree of differentiation	*0.82*	*0.44-1.53*	*0.5*	*0.99*	*0.50-1.94*	*0.9*
KRAS status	*0.66*	*0.41-1.09*	*0.1*	*0.64*	*0.38-1.11*	*0.1*
RIPK3 expression	*0.61*	*0.38-0.97*	*0.044*	*0.60*	*0.37-0.98*	***0.045***

Table 5. Results of Cox regression analysis for predicting overall survival

Index	Univariate analysis			Multivariate analysis		
Variable	*Hazard ratio*	*95% Cl*	*P-value*	*Hazard ratio*	*95% Cl*	*P-value*
Age	*0.84*	*0.50-1.41*	*0.5*	*0.74*	*0.43-1.26*	*0.2*
Gender	*0.76*	*0.45-1.29*	*0.3*	*0.84*	*0.49-1.46*	*0.5*
Degree of differentiation	*0.32*	*0.16-0.62*	***<0.001***	*0.33*	*0.16-0.68*	***0.003***
KRAS status	*1.35*	*0.80-2.27*	*0.2*	*0.89*	*0.50-1.57*	*0.6*
RIPK3 expression	*0.59*	*0.35-0.98*	*0.04*	*0.65*	*0.38-1.11*	*0.1*

7. Discussion

Advanced metastatic CRC remains an incurable disease that still is a challenge to the multidisciplinary team of clinicians, surgeons and chemotherapists due to the high incidence of the disease in the population, relatively late diagnosis, high incidence of distant dissemination in the liver and other organs, lack of sufficient predictive markers for response to treatment and available drug resistance.

The introduction and widespread use of targeted agents has led to significant improvements in progression-free survival and overall survival in patients with metastatic colon cancer. Despite advances in the treatment of these patients, as well as a better understanding of molecular pathways, knowledge of why some patients respond better to certain treatments than others remains insufficient. Although great advances and advances in medicine and oncology, the need to discover new predictive and prognostic factors remains key in terms of patient survival and quality of life.

Necroptosis is a type of programmed cell death that is involved in a number of biological processes such as inflammation, immune response, metabolic processes and cancer. Among other proteins, RIPK3 plays a major role in necroptotic signaling. Resistance to regulated cell death is one of the hallmarks of cancer - it maintains cell survival and significantly limits the effectiveness of conventional drug therapy. SN38, a topoisomerase inhibitor, has been shown to stimulate necroptosis progression, inhibit cell proliferation, and induce the accumulation of DNA damage in colon cancer. These findings suggest that inhibiting the activity of the components of necroptosis may be a strategy in the treatment of cancer. Given the growing importance of necroptosis in many neoplastic processes, a better understanding of the molecular mechanisms underlying necroptotic signaling is likely to have important implications for the development of new methods for regulating necroptosis in tumorigenesis, as well as provide

information on the development of strategies for overcoming therapeutic resistance.

RIPK3 is a proven independent prognostic marker in a large number of cancers such as low-grade gliomas, where high levels of RIPK3 expression correlate with increased mortality. A number of studies have also shown an association between low RIPK3 expression and a worse prognosis in patients with breast cancer, stomach cancer, ovarian cancer, and squamous cell carcinoma of the cervix.

Results from a retrospective study by Ke-jie Wand et. al, which investigated the role of RIPK3 as a prognostic factor in prostate cancer patients, showed that RIP3 was significantly reduced in cell lines and prostate cancer clinical trials and clinical trials, while RIP3 overexpression inhibited prostate cancer migration and invasion. These findings suggest that RIP3 is responsible for the progression of prostate cancer, suggesting that RIP3 may have the potential to be a prognostic marker or therapeutic target against prostate cancer.

Other data show that patients with squamous cell carcinoma of the lung (SCC NSCLC) with low RIPK3 expression have an approximately 2 to 3-fold increased risk of death compared to those with high RIPK3 expression. This observation indicates that, as found in patients with esophageal SCC, RIPK3 inhibits tumor progression in patients with the same histological type in NSCLC. Patients with low RIPK3 expression and high p53 showed an approximately 8.4-fold increased risk of death compared with those with high RIPK3 expression and low p53. This result suggests that the use of combinations of biomarkers may help to better classify patients according to their prognosis.

For the first time in Bulgaria and the world in our study we looked at the relationship between the expression levels of the marker for necroptosis RIPK3 and clinicopathological characteristics of patients such as gender, KRAS status, degree of differentiation and the relationship between OS and PFS in patients with mRCC. Our

retrospective follow-up included 74 patients with CRC in the metastatic stage who underwent a minimum of 3 courses of first-line 5-FU-based systemic chemotherapy. Despite the relatively small group of patients studied and the need for further studies, the data suggest that the level of RIPK3 expression in primary tumors is a new independent potential predictive biomarker for progression-free survival in patients with metastatic colon cancer.

We also found a link between the essential necroptosis marker RIPK3 and clinical benefit (CBR). According to our results, high RIPK3 expression is associated with better OS, making RIPK3 a potential prognostic marker in patients with mRNA. According to the results of our study, no significant relationship was found between the novelty of RIPK3 expression and the clinicopathological characteristics of patients such as gender, KRAS status, degree of differentiation.

Following the publication of our results regarding the role of RIPK3 as a prognostic factor in patients with advanced CRC, other worldwide studies support our claims. Result of a retrospective study from April 2021. of Qun Zhao et. Al, including a larger patient cohort of 41 normal black samples and 286 tumor samples from CRC patients similar to ours, showed that overall survival was significantly higher in patients with high RIPK3 expression than in patients with reduced expression of RIPK3 in colorectal cancer.

5-FU, an analogue of pyrimidine uracil, where hydrogen at position C5 is replaced by fluorine, is an antineoplastic drug that acts as an antimetabolite. It is one of the most commonly used chemotherapeutic agents, and in combination with other drugs is included in drug regimens for the treatment of CRC, carcinomas of the head and neck, cancer of the stomach, breast and others. Due to its structure, 5-FU interferes with nucleoside metabolism and can be incorporated into RNA and DNA, leading to cytotoxicity and cell death. The good effect of 5-FU-based chemotherapy is largely related to the expression levels of several genes, such as TS and DPD. Studies show that 5-FU exerts its anti-cancer effects mainly by 5-FU, an analogue of pyrimidine

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Several studies are currently underway to increase the sensitivity of 5-FU in cancer cells and increase its therapeutic efficacy through the use of new combination therapies. The combination of 5-FU and other therapeutic agents increases the drug response rates in several cancers. However, the role of TME in cancer progression and the development of de novo resistance is also critical. Antiangiogenic therapy targeting angiogenic growth factors (VEGF) and TME-related genes has shown better results when combined with 5-FU chemotherapy. Understanding the mechanisms of resistance, as well as discovering new prognostic and predictive factors in response to 5-FU treatment, is an essential step in overcoming this problem.

Although the statistical significance of our study is limited by the small size of the patient cohort, the current retrospective study suggests that patients with high RIPK3 expression in the primary tumor may have a better effect with 5-FU based first-line chemotherapy. This is evidence presented for the first time in Bulgaria

and the world, but further research is needed to investigate in more detail the role of necroptosis in patients with CRC. Despite these limitations, detecting the level of RIPK3 expression may have the potential to identify suitable patients for new pronecroptotic therapy in clinical trials to improve patient outcomes and survival.

7. Summary

In conclusion, as a summary of our retrospective study, it is clear that the necroptosis marker RIPK3 may serve as an independent prognostic marker in terms of overall survival in patients with advanced mCRC.

The number of studies available to date on the relationship between necroptosis, its markers and mRCC is relatively small, so more research is needed to elucidate the exact molecular mechanisms of interaction between different types of cell death and their role in neoplastic processes. This would provide information for building better therapeutic strategies for the treatment of patients with malignancies and in particular those with CRC.

8. Conclusion

1. There is no correlation between the level of RIPK3 expression in primary tumors and the clinicopathological characteristics of patients with CRC in the metastatic stage.

2. Better overall survival was found in patients with high RIPK3 expression, making it a potential prognostic marker.

3. A better response to 5-FU based first-line chemotherapy has been found in patients with higher RIPK3 expression.

4. The potential prognostic value of RIPK3 as a predictive factor for 5-FU based chemotherapy was established.

5. There was no statistically significant association between RIPK3 expression level and progression-free survival in patients with mCRC.

9. Contributions

1. For the first time in Bulgaria the connection between the level of expression of the marker for necroptosis RIPK3 in patients with metastatic CRC is studied.

2. For the first time in Bulgaria, the potential for the level of RIPK3 expression to be used as a prognostic factor in terms of overall survival in patients with colon cancer has been reported.

3. For the first time in Bulgaria, the potential of the level of RIPK3 expression to be used as a predictive factor for 5-FU-based first-line chemotherapy in patients with mCRC has been reported.

4. For the first time in the world literature, the relationship between the necroptosis marker RIPK3 and the response of patients to treatment with 5-FU based first-line chemotherapy in patients with mCRC has been studied and reported.

10. Publications releated to the dissertations

1. „Автофагия при солидни тумори“.
Автори: Елеонора Димитрова, Иван Донев, Николай Цонев, Соня Драганова, Ростослав Манев, **Маргарита Богданова**, Христо Попов, Надежда Стефанова, Драгомир Стоянов, Явор Кашлов, Асен Янчев, Маргарита Георгиева.
Издание: Studia Oncologica
Страници: 47-56.
Дата на публикуване: 2016
Издател: Парадигма

2. „Некроптоза“
Автори: **М. Богданова**, И. Донев, Н. Цонев, Е. Димитрова, Р. Манев, Др. Стоянов, Ч. Бъчваров, Г. Тодоров, Т. Радев, Н. Стефанова, К. Калчев, М. Таушанова
Издание: Studia Oncologica
Страници: 59-68
Дата на публикуване: 2017.
Издател: Парадигма

3. „Некроптоза - молекулярни механизми и ролята й при злокачествените тумори“
Автори: Надежда Стефанова, Мария Цанева, Калин Калчев, **Маргарита Богданова**, Иван Донев
Издание: Варненски медицински форум.
Дата на публикуване: 2017.
Издател: МУ-Варна

4. „RIPK3 expression as a potential predictive and prognostic marker in metastatic colon cancer“

Authors: Conev NV, Dimitrova EG, **Bogdanova MK**, Kashlov YK, Chaushev BG, Radanova MA, Petrov DP, Georgiev KD, Bachvarov CH, Todorov GN, Kalchev KP, Popov HB, Manev RR, Donev IS.
Journal with IF*:* Clinical and Investigative medicine.
Date of publication: 2019/3.

5. „Имунотерапия при колоректален карцином“
Автори: д-р Ростислав Манев, **д-р Маргарита Манева**, доц. д-р Мария Раданова, дм,
доц. д-р Николай Цонев, дм
Издание: МЕДИНФО
Дата на публикуване: 2020/4

6. „Single nucleotide polymorphisms in microRNA genes and colorectal cancer risk and prognosis“
Authors: Maria Radanova, Maria Levkova, Galya Mihaylova, Rostislav Manev, **Margarita Maneva**, Rossen Hadgiev, Nikolay Conev, Ivan Donev
Journal with IF: Biomedicines.
Date of publication: 2022*/1*

7. “ Key Apoptosis Signaling Pathways In Malignant Diseases”
Автори: *Teodorika Panayotova, Dragomir Stoyanov,* ***Rostislav Manev****, Margarita Maneva, Boryana Stefanova, Eleonora Dimitrova, Nikolay Tsonev*
Дата на публикуване: *2021/12/30*
Издание: *Varna Medical Forum*

8. „3-year clinical experience in patients with unresectable metastatic colon cancer received first-line of chemotherapy“

39

Автори: *Rostislav Manev,* ***Margarita Maneva****, Zhasmina Mihaylova, Nikolay Conev*
Издание: *Scripta Scientifica Medica*
Дата на събмитване: *2022/3 (предстоящо публикуване)*
Издател: *МУ - Варна*

Acknowledgments:

Special thanks to my supervisor:

Assoc. Prof. Eleonora Dimitrova, Ph.D. - for help and guidance

Special thanks to:

Assoc. Prof. Dr. Nikolay Tsonev, Ph.D. - for the irreplaceable help and ideas

Prof. Dr. Ivan Donev, Ph.D. - for the idea of a topic, collaboration during the work and the assistance during the whole study

The team of the Clinic of Medical Oncology at the University Hospital "St. Marina ”- Varna and especially to my family for their support and patience !!!