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APOPTOSIS AND NECROPTOSIS IN RENAL CELL CARCINOMA

THESIS SUMMARY

of PhD dissertation

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Prof. Maria Tsaneva, MD, PhD

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The dissertation contains **169** standard pages and is illustrated with 80 tables and 65 figures. The bibliography includes **266** literature sources, **5** are in Cyrillic and **261** in Latin.

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The public defense of the dissertation will take place on 17.01.2025, before a scientific jury composed of:

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Contents

Abbreviations used:

AIF - apoptosis inducing factor
AP - apoptosis
ccRCC - clear cell carcinoma
chRCC - chromophobe carcinoma
CRC - colorectal carcinoma
ISUP - International Society of Urological Pathology
LVI - lymphovascular invasion
MVI - microvascular invasion
NP - necroptosis
pRCC - papillary carcinoma
RCC - renal cell carcinoma
RIPK3 - Receptor interacting protein kinase 3
TILs - tumor infiltrating lymphocytes
TN - tumor necrosis
WHO - World Health Organization

I. INTRODUCTION

Renal cell carcinoma (RCC) incidence is between 1% and 3% of all malignant visceral neoplasms. Approximately 40% of patients with this carcinoma die due to disease progression and therefore it is considered one of the most malignant neoplasms among malignant urological tumors. In most cases, RCC is detected by imaging studies performed due to urological or other complaints. Male gender predominates among patients, about two-thirds of cases (Muglia VF, Prando A, 2015). In Bulgaria, there has been a trend of increasing mortality from kidney and urinary tract tumors per 100,000 people for the period from 1980 to 2017. In women, mortality increased from 0.9 in 1980 to 1.4 in 2017, while in men it increased from 1.6 to 3.8, respectively (Valerianova et al., 2020).

Apoptosis (AP) or programmed cell death is the universal pathway for eliminating unnecessary cells and tissues involving a phagocytosis process without inducing an inflammatory reaction (Greenhalgh DG, 1998). There are two main AP pathways: external and internal (Xu X et al., 2019). Programmed cell death is a coordinated and often energy-dependent process that involves the activation of a group of cysteine proteases called caspases and a complex cascade of events that link initiating stimuli to the final death of the cell (Elmore S, 2007).

AP is considered a major protective mechanism against the occurrence and progression of a number of neoplasms, including renal cell carcinoma (Ganini C et al., 2022). This form of cell death finds application in the field of biomedicine for the destruction of neoplastic cells in response to externally applied stimuli that induce AP, such as small molecule drugs (Xu X et al., 2019). Most chemotherapeutics, as well as radiotherapy, cause cellular damage to tumor cells, activate the internal signaling pathway, but active *p53* is also required for its implementation (Ashkenazi A, 2002).

Apoptosis-inducing factor (AIF) is an apoptogenic mitochondrial intermembrane protein that acts independently of Bcl-2 and caspase control in the cell death process (Susin SA et al., 1999). As a caspase-independent AP mechanism, AIF may be a potential target for chemoradiotherapy in a number of malignancies (Millan A, Huerta S, 2009).

Necroptosis (NP) is an alternative form of cell death that is initiated when the process of AP is inhibited (Degterev A et al., 2005). NP, mediated by receptor-interacting protein kinase-3 (RIPK3) and its substrate mixed lineage kinase domain-like protein (MLKL), is the best characterized form of regulated necrosis (Pasparakis M, Vandenabeele P., 2015). NP is thought to play a key role in multiple aspects of tumor biology, including oncogenesis, tumor metastasis, and tumor immunity (Liu S et al., 2021). It has a dual role in different types of neoplasms, and the mechanisms underlying these effects may depend on the type and stage of the tumor. Aberrant suppression or activation of this type of cell death is closely related to the occurrence and development of neoplastic diseases. These findings may provide new directions in tumor therapy and effective NP regulation in tumor cells in the near future (Yang M et al., 2022).

RIPK3 signaling has both tumor-repressive and tumor-stimulating effects. The role of RIPK3 in the development of various tumors, their progression, metastasis and recurrence may not be unambiguous. The pro-carcinogenesis or anti-carcinogenesis of RIPK3 signaling depends mainly on the balance of cytokines and chemokines produced, as established in some tumors. A more detailed elucidation of the RIPK3 signaling pathway in the pathogenesis of various types of neoplasms, and especially of RCC, is necessary (Liu S et al., 2021).

RCC is characterized by uncontrolled cell proliferation, lack of cell death and strong resistance to conventional chemotherapy (Toth C et al., 2017). According to some authors, in renal carcinoma

there are damages to the internal and external signaling pathway of AP (Toth C et al., 2017).

In different types of tumors, including RCC, there are complex interactions between different cell death signaling pathways. Given the pronounced resistance of renal carcinoma to standard therapeutic methods, it is necessary to search for new approaches in its treatment by influencing the signaling pathways of AP and NP, taking into account the interactions between them, as well as their connection with the clinical and morphological indicators of the tumor.

II. AIM AND OBJECTIVES

2.1. Aim

The aim of the present study is to investigate the immunohistochemical expression of the apoptosis marker, apoptosis-inducing factor (AIF) and necroptosis marker, Receptor-interacting protein kinase 3 (RIPK3) in patients with renal cell carcinoma and to determine their prognostic value.

To achieve the set aim, the following objectives were formulated:

2.2. Objectives

1. To study and compare the clinical and morphological characteristics in relation to the survival of selected RCC patients.
2. To determine the immunohistochemical expression of AIF in RCC tumor tissue and compare it with the adjacent non-tumor tissue.
3. To evaluate semi-quantitatively the immunohistochemical expression of RIPK3 in RCC tumor tissue and compare it with the adjacent non-tumor tissue.

4. To evaluate the immunohistochemical expression of AIF and RIPK3 in metastatic lesions of selected cases with histologically verified metastases and compare it with the expression in the primary tumor.
5. To investigate the immunohistochemical expression of AIF and RIPK3 in relation to the clinical and pathological characteristics of RCC patients with renal cell carcinoma: gender, age, tumor stage, histological type, degree of differentiation, tumor necrosis, tumor-infiltrating lymphocytes and vascular invasion.
6. To analyze the apoptosis and necroptosis markers, AIF and RIPK3, in relation to patient survival and determine their prognostic role in renal carcinoma.

III. MATERIAL AND METHODS

3.1. Materials used in the study

3.1.1. Facilities and data used in the dissertation

- Department of General and Clinical Pathology, Forensic Medicine and Deontology, Medical University of Varna
- Data from Saint Marina University Hospital, Varna's electronic database MultiLab was used.

3.1.2. Patient population

The study included 80 RCC patients, divided into three groups:
First group: 20 patients, diagnosed with papillary renal cell carcinoma.
Second group: 21 patients, diagnosed with chromophobe renal cell carcinoma.
Third group: 39 patients diagnosed with clear cell renal cell carcinoma.

All patients were operated on at Saint Marina University Hospital, Varna. Histological preparations from the primary tumors were examined and the following histological indicators were evaluated: histological type, presence and area of tumor necrosis, TILs, vascular invasion and degree of differentiation in clear cell and papillary carcinoma. The TNM stage was determined. In 15 of the patients, there were histologically verified distant metastases.

The levels of immunohistochemical expression of AIF and RIPK3 were analyzed in the three patient groups and in fourteen histologically verified distant metastases.

3.2. Research methods

3.2.1. Histological studies

From each tumor resected specimen, an average of three to four materials were examined, including tumor parenchyma, foci with necrosis and adjacent non-tumor tissue, as well as histologically verified metastases. The materials were fixed in 10% neutral buffered formalin and after appropriate processing were included in paraffin with a melting point of 52-54ºC in order to prepare paraffin blocks. Sections with a thickness of 5 μm were routinely stained with hematoxylin-eosin to assess histological changes in the primary tumor and metastases.

Criteria for categorizing each indicator:

- ***Age.*** Patients were divided into three age groups: ≤ 44 years, 45-64 years and ≥ 65 years.
- ***Gender.*** Patients were divided into two gender categories, male and female.
- ***Tumor localization***. Tumors were categorized according to their localization in the left or right kidney to compare with results from other studies.

- ***Histological tumor type.*** It was determined according to the WHO criteria for kidney tumors from 2016 (Moch H et al, 2016) and 2022 (Amin MB et al., 2022).
- ***TNM stage.*** RCC stage was based on the WHO classification from 2022. Presence of lymph node metastases (N) and distant metastases (M) was established by biopsy examination.
- ***Degree of differentiation.*** The four-grade ISUP scale was used, based on visualization of nucleoli at different microscope magnifications, as well as pleomorphism, rhabdoid or sarcomatoid differentiation and multinucleated tumor cells. According to current WHO recommendations, the degree of differentiation is not determined in chromophobe carcinoma.
- ***Necrosis area.*** Necrosis was determined semi-quantitatively, using a four-point scale: Group I - absent; Group II - focal, <10% of the tumor tissue area; Group III - moderately expressed necrosis, from 10% to 30% of the tumor tissue area; Group IV - extensive necrosis, ≥30% of the tumor tissue area.
- ***Tumor infiltrating lymphocytes (TILs).*** TILs include intratumoral and stromal TILs. Intratumoral TILs are lymphocytes in tumor nests, in contact with carcinoma cells without intervening stroma. Stromal TILs are in the tumor stroma and do not directly contact tumor cells (Salgado R, et al., 2015). An average of two to three preparations from each case were analyzed to determine the intensity (I) of TILs in tumor tissue and/or stroma (Zhang D al., 2019) on the scale: 0 - absent; 1- slight increase in TILs (weakly expressed); 2 - increased TILs (moderately expressed); 3 - prominent TILs (expressed).
- ***Vascular invasion (LVI).*** The presence or absence of LVI was reported in vessels of various calibers, with an average of

three to four sections from each tumor resected specimen being evaluated.

3.2.2. Specific Research Methods

Immunohistochemical method and antibodies used.

An indirect immunoperoxidase method for immunohistochemical analysis was applied using mini KIT high Ph DAKO K8024. The following antibodies were used:

- Recombinant Anti-AIF antibody (E20) - Mitochondrial Marker cat. №ab32516, monoclonal rabbit antibody, as apoptosis marker (ABCAM's RabMab technology).
- Anti-RIPK3 antibody cat. № ab62344, polyclonal rabbit antibody, necroptosis marker (ABCAM's RabMab technology).

Antibody expression levels were determined using an HRP/DAB anti-polyvalent detection system. The reaction was visualized using an appropriate substrate chromogenic reagent (DAB/Diaminobenzidine). The antibodies, staining reagents, and working concentrations used are presented in Table 1.

Table 1. *Reagents used.*

Antibody	Dilution	Positive control	Marker for:	Manufacturer
Recombinant Anti-AIF antibody [E20] - Mitochondrial Marker (ab32516) Rabbit monoclonal [E20] to AIF	1:500	Squamous cell cervical carcinoma	Apoptosis	ABCAM's RabMab technology
Anti-RIPK3 antibody (ab62344) Rabbit polyclonal to RIPK3	1:300	Kidney	Necroptosis	ABCAM's RabMab technology

Method of reporting AIF and RIPK3 expression

The assessment of immunohistochemical expression was made by examining 10 fields at the highest magnification (x 400) for each individual case.

The immunohistochemical expression of AIF/RIPK3 was evaluated semi-quantitatively using H-score (histo-score) on tissue sections. First, the cytoplasmic or nuclear intensity (0, 1+, 2+, or 3+) was determined for each cell in different fields. The percentage of positive cells for each separate intensity was calculated, and finally, the H-score was calculated using the following formula (Ishibashi H et al., 2003):

[1x (% cells with 1+) + 2x (% cells with 2+) + 3x (% cells with 3+)], ranging from 0 to 300.

H-score was also used to evaluate nuclear expression of AIF/RIPK3. The result was evaluated using the formula:

[1x (% nuclei with 1+) + 2x (% nuclei with 2+) + 3x (% nuclei with 3+)], ranging from 0 to 300.

3.2.3. Statistical Methods

The following methods were applied for statistical data processing:

A. Descriptive analysis:

-Variation analysis

-Alternative analysis

-Checking the normality of data distribution was done graphically and quantitatively

B. For hypothesis testing, the following methods were used:

1. Parametric methods

2. Non-parametric methods

A significance level of alpha = 0.05 was adopted for the null hypothesis. All statistical tests were two-sided.

C. Tabular and graphical methods for illustrating the obtained results.

Data processing and analysis were performed with the statistical package IBM SPSS ver. 21, and the graphs were constructed in MS Excel for Windows.

IV. RESULTS AND DISCUSSION

4.1. Clinical and morphological characteristics of RCC patients.

The mean age of RCC patients was 62.8 years (SD 10.9). The youngest was 28 years old, and the oldest was 81 years old. Half of the patients were between 54 and 70 years old.

Our results (Fig. 1) showed that the largest number of RCC patients was in the age group 60-69 years. According to literature data, most diagnosed RCC cases were between the ages of 60 and 70 (Capitanio U et al., 2019; Liao Z et al., 2022), with a mean age of 64

years (Rosiello G et al., 2021), which is consistent with the data we obtained.

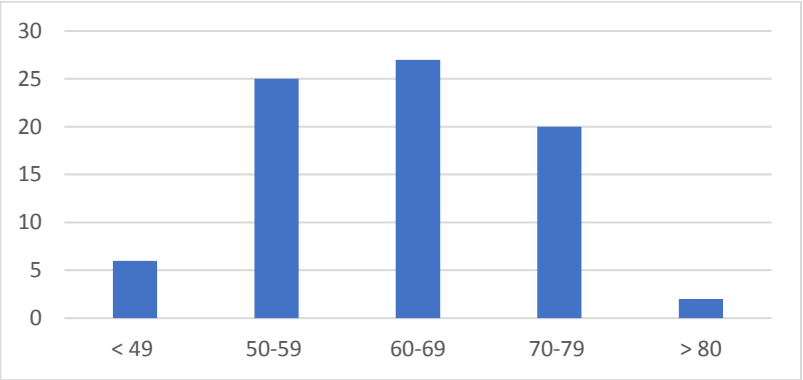


Figure 1. *Patient distribution by age.*

There were 4 patients in the first group ≤ 44 years, 40 in the second group from 45 to 64 years, and 36 in the third group ≥ 65 years.

Table 2 presents the clinical and morphological characteristics of the studied RCC patients.

We found that RCC occurs more frequently in males - 56 (70%) cases, compared to females - 24 (30%) cases. Our data do not differ from those published in the literature, according to which this carcinoma occurs more frequently in men (Scelo G et al., 2018). According to Scelo et al. (2018), the male:female ratio for this disease is 2:1 and it is not dependent on age, year of study, and region, which suggests that factors other than socio-cultural and health behavior are related to gender differences in RCC. Other authors also report the predominant involvement of males and a similar ratio between the two genders (Guo S et al., 2019), including ccRCC (Feng X et al., 2019).

Histological examination of RCC revealed that 39 (48.8%) were clear cell carcinomas, 20 (25%) had papillary

characteristics, and 21 (26.2%) were chromophobe (Fig. 2). According to WHO data from 2022, these are the three most frequently diagnosed histological variants of RCC, occurring with frequencies of 60-75%, 13-20%, and 5-7%, respectively (Amin et al., 2022).

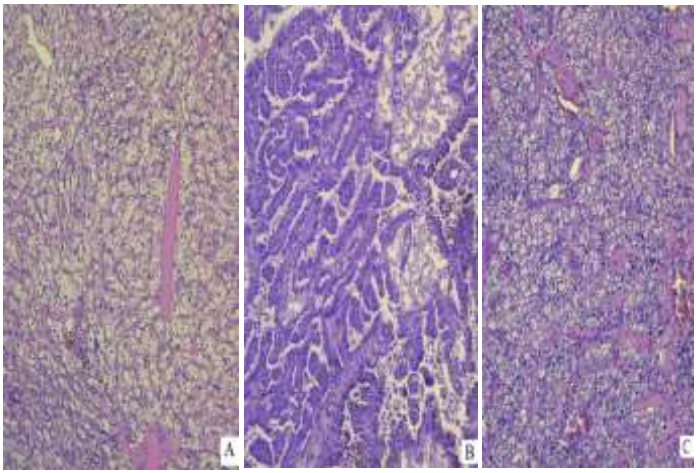


Figure 2. *A. Clear cell renal carcinoma, HE x 100; B. Papillary renal carcinoma, HE x 100; C. Chromophobe carcinoma, HE x 100.*

Table 2. *Clinical and morphological characteristics of the 80 studied RCC patients.*

Characteristics	**Number (n)**	**%**
Total number	80	100
1. Gender		
Male	56	70.0
Female	24	30.0
2. Histological type		
Clear cell	39	48.8
Papillary	20	25.0
Chromophobe	21	26.2
3. Differentiation /ISUP/		
G1	3	3.8
G2	28	35.0
G3	21	26.2
G4	7	8.8
Gx	21	26.2
4. Necrosis area		
no /I group/	35	43.8
> 10 % /II group /	16	20.0
10-30 % /III group/	16	20.0
> 30 % /IV group/	13	16.2
5. Tumor infiltrating lymphocytes /TILs/		
Absent	50	62.5
Weakly expressed	21	26.3
Moderately expressed	9	11.2
Expressed	0	0
6. Vascular invasion (LVI)		
Absent	47	58.8
Present	33	41.2
7. T stage		
T1	35	43.8
T2	14	17.5
T3	26	32.5
T4	5	6.2
8. N stage		
N0	19	23.8
N1	9	11.2
Nx	52	65.0
9. M stage		
M0	3	3.7
M1	15	18.8
Mx	62	77.5
10. Localization in kidney		
left	42	52.5
right	38	47.5

In the USA, Feng et al. (2019) analyzed clear cell renal carcinoma over a period of 40 years (from 1973 to 2014) and found that its frequency increased with age, reaching a peak in individuals aged between 60 and 79 years, after which its frequency decreased.

In the microscopic analysis of papillary and clear cell carcinomas, the degree of differentiation (G) was also determined (Fig. 3), using the ISUP system specified in the WHO classification from 2016 (Moch H et al. 2016) and from 2022 (Amin MB et al., 2022).

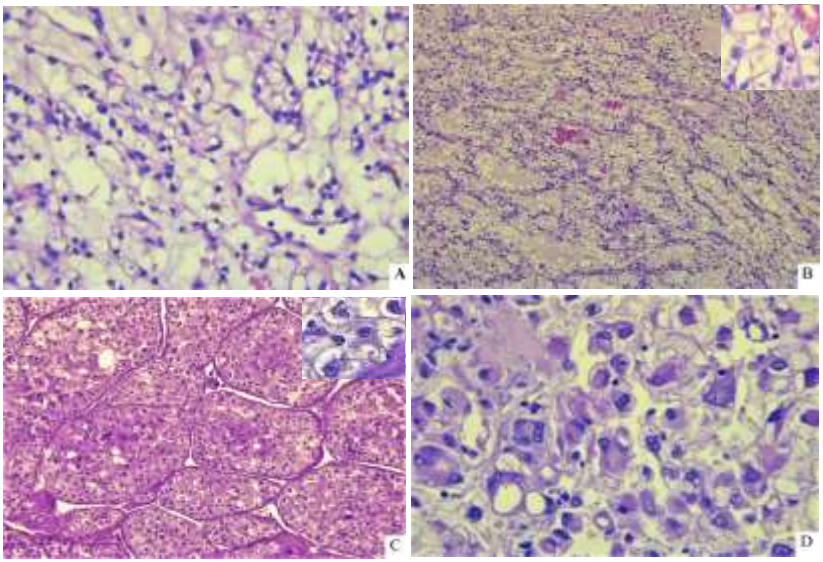


Figure 3. *Degrees of differentiation of RCC according to the ISUP scale:*
A)G1 - Nucleoli are absent or inconspicuous, HE x 400;
B)G2 - Nucleoli are not evident at 100x magnification; HE x100;
C)G3 - Nucleoli are noticeable and eosinophilic at 100x; HE x100,
D)G4 - Nuclear pleomorphism, multinucleated cells, rhabdoid differentiation, HE x 400.

We found that 3 (3.8%) cases had G1 differentiation, 28 (35.0%) cases G2, 21 (26.2%) cases G3, and 7 (8.8%) cases - G4. The degree of differentiation was not determined in 21 (26.2%) cases of chromophobe carcinoma, according to the latest WHO recommendations (Amin MB et al., 2022), as differentiation in this histological variant has no prognostic value (Ohashi R et al., 2020).

Regarding tumor necrosis, it was found that 35 (43.8%) of the cases fall into Group I, where necrosis was absent, 16 (20%) patients each into Groups II and III, and 13 (16.2%) of the RCC cases into Group IV (Fig. 4 A and 4 B).

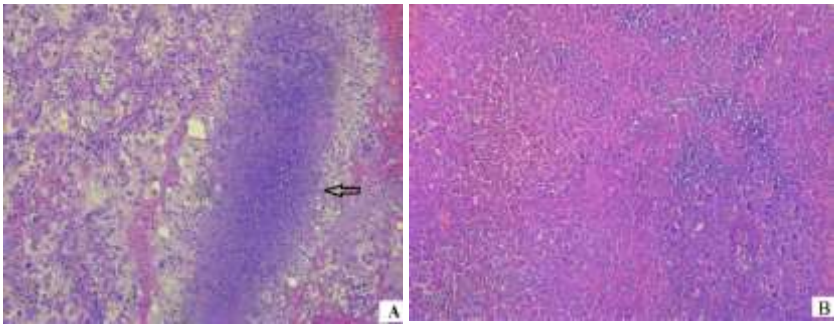


Figure 4. *Types of necrosis in tumor tissue in RCC according to their area A). Weakly expressed necrosis, HEx100; B). Moderately expressed necrosis, HE x100.*

TILs in tumor tissue were also noted in all RCC cases (Fig. 5A and Fig. 5B). In 50 (62.5%) cases, TILs were absent, in 21 (26.3%) they were weakly expressed, in 9 (11.2%) they were moderately expressed, and in none of the cases were they expressed (0%).

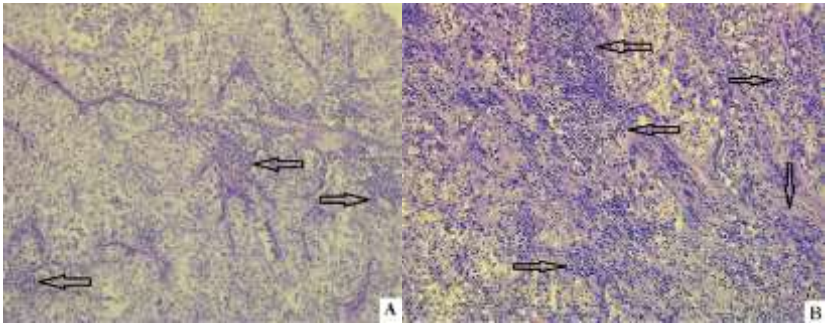


Figure 5. *RCC with A) weakly expressed, HE x 100 and B) moderately expressed infiltration of tumor-infiltrating lymphocytes, HE x 100.*

We found LVI in vessels of various calibers in 33 (41.2%) of the cases, while LVI was absent in 47 (58.8%) of the examined RCCs.

RCC is one of the best-vascularized tumors, with branched, thin-walled vessels between tumor nests being a characteristic feature of ccRCC, and therefore it is not surprising that vascular invasion is often found in these tumors (Delahunt B et al., 2013). RCC with vascular invasion has high mortality (Rodríguez-Cabello MA et al., 2017). According to literature data, the frequency of vascular invasion varies between 16.7% and 29% (Lang H et al., 2004; Madbouly K et al., 2007). Differences in frequency may be due to the different number of patients included in the studies, as well as the number of sections examined from each tumor.

According to the T stage of RCC, the cases were distributed as follows: 35 (43.8%) cases in stage T1, 14 (17.5%) in T2, 26 (32.5%) in T3, and 5 (6.2%) in stage T4 (Table 2). Data from a study by Guo et al. (2019), including 41,138 operated patients with RCC, found that cases in T1 stage were 68.0%, in T2 - 10.4%, in T3 - 20.3%, and in T4 - 1.3%. The data we obtained differ from those of Guo et al. (2019), which is most likely due to the selected cases in the present study.

Analyzing the N stage of RCC, we found that in 52 (65.0%) patients there were no histologically examined lymph nodes (Nx), in

19 (23.8%) cases there were no metastases in the lymph nodes (N0), and in 9 (11.2%) cases there were metastases (N1).

In the study by Guo et al. (2019), 96.8% of cases had no metastases (N0), and only 3.2% had metastases in the regional lymph nodes (N1).

Regarding distant metastases, we found that for 62 (77.5%) of the patients there was no data on distant metastases and they were staged as Mx. In three (3.7%) patients there were no metastases (M0), and in 15 (18.8%) there were histologically verified metastases (M1). Organs in which histologically proven distant metastases were found included adrenal gland, brain, vertebrae, lung, skin, gum mucosa, sternum, and muscle.

According to Bianchi et al. (2012), some of the most common locations of distant metastases in RCC were lung, bones, liver, adrenal glands, and brain. Taken together, our results and data from the literature show that distant metastases can be found in various organs of the human body.

According to tumor localization, we found that in 42 (52.5%) of the patients, the tumor was located in the left kidney and in 38 (47.5%) in the right kidney. In the study by Guo et al. (2019), in 50.6% of cases the tumor was in the right kidney, and in 49.4% in the left kidney. According to the authors (Guo S et al., 2019), the right-sided location of RCC was associated with an earlier stage, higher degree of differentiation, and showed better tumor-associated survival compared to left kidney localization. Similar dependencies were not analyzed in the present study due to the selection of the studied patients.

4.2. Comparative analysis between different clinical-morphological indicators in renal cell carcinoma

4.2.1. Relationship between T stage and clinical-morphological indicators

Relationship between T stage and tumor differentiation

We analyzed the T stage of the tumor in relation to the differentiation of ccRCC and pRCC and found a significant relationship (χ^2=21.278, p=0,017). To verify the results, we divided the tumors into two groups according to the degree of differentiation, corresponding to low-grade malignancy (G1 and G2) and high-grade malignancy (G3 and G4), and again compared them with T stage (Table 3). The difference was again statistically significant (χ^2=10.897, p=0,012). It is notable that as the degree of differentiation decreases, the number of carcinomas in advanced stages (T3 and T4) increases.

Table 3. *Distribution of RCC cases by degree of differentiation and T stage*.

Group	T1	T2	T3	T4	Total
G1,G2	18(58,1%)	5(16,1%)	7(22,6%)	1 (3,2%)	31(100%)
G3,G4	6 (21,4%)	3 (10,7%)	16(57,1%)	3(10,7%)	28(100%)
Total	24 (40,7%)	8 (13,6%)	23(39,0%)	4 (6,8%)	59(100%)

Our data did not differ from those obtained by Spasova (2018) for metastatic RCC. She found a statistically significant correlation between tumor size and high nuclear grade, determined by the Fuhrman system in tumor tissue. Larger tumors were associated with lower differentiation (Spasova S, 2018).

Relationship between T stage and necrosis area

A statistically significant relationship was found between T stage and the extent of TN spread (χ^2=25.148, p=0,003) (Table 4). High T stage correlates with a large area of TN.

***Table 4**. Distribution of RCC cases by T stage and extent of TN spread.*

Group	No necrosis	Necrosis < 10%	Necrosis 10-30%	Necrosis > 30%	Total
T1	23(65,7%)	7 (20,0%)	1 (2,9%)	4 (11,4%)	35(100%)
T2	6 (42,9%)	4 (28,6%)	4 (28,6%)	0 (0,0%)	14(100%)
T3	6 (23,1%)	4 (15,4%)	9 (34,6%)	7 (26,9%)	26(100%)
T4	0 (0,0%)	1 (20,0%)	2 (40,0%)	2 (40,0%)	5 (100%)
Total	35(43,7%)	16(20,0%)	16(20,0%)	13(16,3%)	80(100%)

In RCC, Lam et al. (2005) also found that the extent of necrosis in the primary tumor significantly correlates with tumor size. A similar relationship between the two indicators was observed by Spasova (2018). The author indicates that there is a statistically significant, direct correlation between tumor size and necrosis spread, but only in the group of non-metastatic RCCs. No such relationship was found in the group of metastatic carcinomas (Spasova S, 2018). Leibovitch et al. (2001) also studied necrosis in kidney cancer, finding that tumors with extensive necrosis were significantly larger and had more frequent perirenal and venous involvement compared to tumors without necrosis. According to the same authors, extensive TN in RCC does not appear to be related to tumor biology, but rather reflects the relationship between tumor size and vascularization (Leibovitch I et al., 2001).

Relationship between T stage and tumor-infiltrating lymphocytes

We analyzed the T stage of RCC versus the four degrees of TILs intensity in tumor tissue and found a statistically significant relationship between the two indicators (χ^2=13.515, p=0,036). A relationship was also found when comparing T stage and data on the presence or absence of TILs. Data analysis showed that the higher the T stage, the more frequent the presence of TILs (χ^2= 5.873, p=0,015) (Fig. 6).

Our results differ from those published in the literature. In the study by Fuchs et al. (2020), which included 1034 RCCs, TILs were divided into three categories: low (0% to 10%), moderate (15% to 50%) and high (55% to 100%). Comparative analysis between TNM stage and TILs, performed by χ^2 test, showed that more intense TILs infiltration was observed at lower TNM stage of RCC. The differences between the present study and that by Fuchs et al. (2020) may be due to both different criteria in determining TILs and tissue conditions.

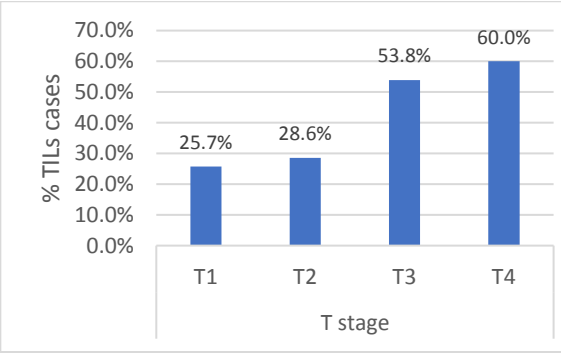


Figure 6. *Relationship between T stage and presence of TILs in RCC cases.*

Relationship between T stage and vascular invasion

We found a relationship between the T stage of the studied RCC patients and LVI in vessels of different calibers, with the highest frequency of LVI cases at stage T4 (χ^2= 33.662, p<0,001) (Fig. 7).

Van Poppel et al. (1997) examined 180 RCC patients after nephrectomy. In their patients, 51 (28.3%) had microvascular invasion, while 129 (71.7%) lacked it on microscopic examination. The researchers found a significant statistical relationship between the two indicators: T stage and MVI. None of the T1 stage cases and approximately one-third of T2 stage cases had no MVI, while

approximately 50% of T3 and T4 stage cases had MVI. According to Van Poppel et al. (1997), although the data show a greater influence of MVI on T2 stage than T3, they also show that microscopic vascular invasion is an independent prognostic factor. According to the same authors, larger tumors have MVI more often than smaller ones. In 121 out of 129 RCC patients (94%) without microscopic vascular invasion, there was no tumor progression, while progression was absent in only 31 out of 51 patients (61%) with vascular invasion (Van Poppel H et al., 1997).

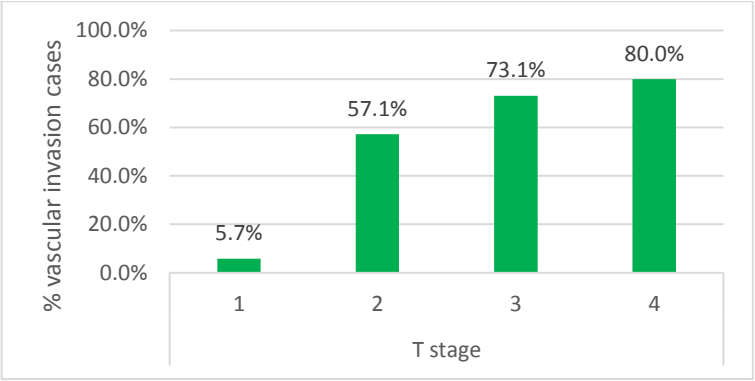


Figure 7*. Relationship between T stage and LVI in RCC patients.*

No statistically significant relationship was found between T stage and other clinical-morphological indicators such as: age and sex of patients, histological variant, N and M stage and RCC localization.

4.2.2. Analysis of tumor necrosis in relation to clinical-morphological indicators

Relationship between tumor necrosis and histological variant of carcinoma

A statistically significant relationship was found between the extent of TN spread and the histological variant of RCC (χ^2=18.632, p=0,005). It is noteworthy that in chRCC, cases without TN predominated, while in pRCC cases without necrosis were the least numerous (Table 5).

When we compared our own results with those from another study (Sengupta S et al., 2005), we found some similarities. In their study, Sengupta et al. (2005) included 3009 RCCs, reporting coagulative TN and defining it in two categories: absent and present. The authors report that in most chRCC cases necrosis was absent, which corresponds to our results. In the present study, ccRCC and pRCC with TN predominated (Table 5), while in the study by Sengupta et al. (2005), in slightly more than half of the ccRCC (72%) and pRCC (53%) cases, necrosis was absent. Our data also differ from other studies (Pichler M et al., 2012). Pichler et al. (2012) also examined TN in RCC and found that it occurred in 33.9% of the 2,285 cases studied, with ccRCC and pRCC cases without TN predominating, while in our study predominated those with necrosis. The presence of TN was an independent predictor of overall survival in patients with ccRCC and pRCC (Pichler M et al., 2012).

Table 5. *Distribution of RCC patients by tumor histological variant and TN spread.*

Group	**no necrosis**	**necrosis < 10%**	**necrosis 10-30%**	**necrosis > 30%**	**Total**
pRCC	4 (20,0%)	5 (25,0%)	3 (15,0%)	8 (40,0%)	20 (100%)
chRCC	15 (71,4%)	2 (9,5%)	3 (14,3%)	1 (4,8%)	21 (100%)
ccRCC	16 (41%)	9 (23,1%)	10 (25,6%)	4 (10,3%)	39 (100%)
Total	35 (43,7%)	16 (20,0%)	16 (20,0%)	13 (16,3%)	80 (100%)

Relationship between tumor necrosis and vascular invasion in RCC patients

We found a relationship between the extent of TN spread and vascular invasion. The larger the TN area, the higher the frequency of cases with LVI (χ^2=6.843, p=0,009) (Fig. 8).

Our results do not differ from those published by Pichler et al. (2012) in their study on RCC. The authors found a statistically significant relationship between TN and vascular invasion in ccRCC and pRCC. A similar relationship between TN and vascular invasion was also found by Klatte et al. (2009) in ccRCC. As the degree of necrosis increased, so did vascular invasion.

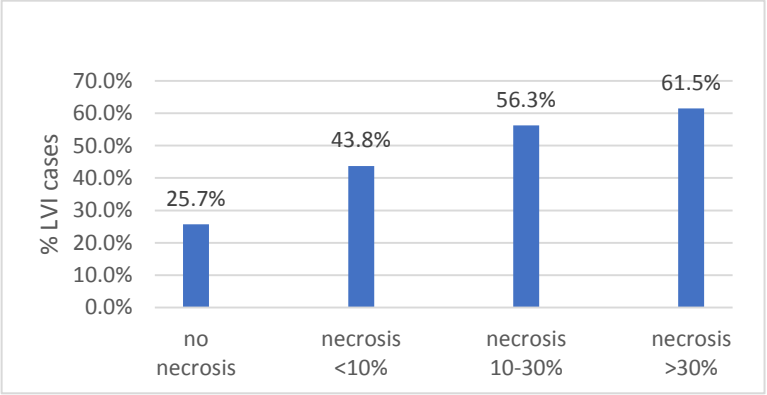


Figure 8. *Relationship between TN area and presence of LVI in RCC patients.*

Relationship between tumor necrosis and RCC localization

We analyzed the extent of TN spread in relation to tumor localization and found a significant statistical relationship (χ^2=9.253, p=0,026). There were more cases with necrosis over 30% in the right kidney, compared to the left kidney (Table 6).

***Table 6**. Distribution of RCC patients by tumor localization in the kidney according to TN spread.*

Group	no necrosis	necrosis < 10%	necrosis 10-30%	necrosis > 30%	Total
left	19 (45,2%)	6 (14,3%)	13 (31,0%)	4 (9,5%)	42 (100%)
right	16 (42,1%)	10 (26,3%)	3 (7,9%)	9 (23, 7%)	38 (100%)
Total	35 (43,7%)	16 (20,0%)	16 (20,0%)	13 (36,3%)	80 (100%)

Tumor necrosis area did not correlate with age, sex, TILs and N and M-stage of RCC patients.

4.2.3. Analysis of tumor-infiltrating lymphocytes and vascular invasion in relation to clinical-morphological indicators

Relationship between tumor-infiltrating lymphocytes and sex of patients

We analyzed TILs in relation to sex of patients included in the study (Fig. 9). A statistically significant relationship was found (χ^2= 12.565, p=0,002), with almost 92% of tumors in females having no TILs, while in males this proportion is 50%.

Our results differed from those observed in CRC (Fuchs TL et al., 2020). The authors found no relationship between TILs in tumor tissue and sex of patients.

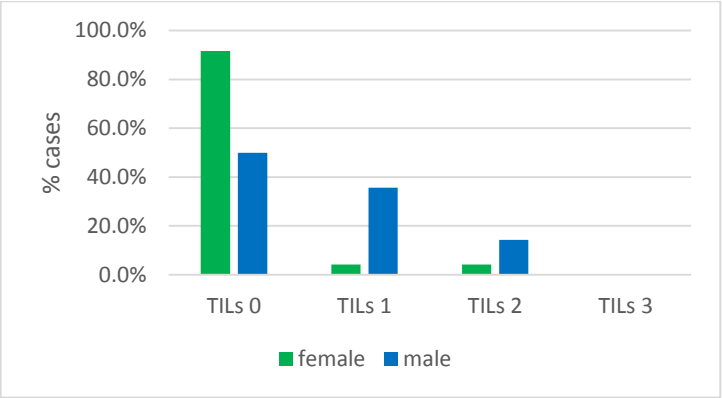


***Figure 9**. Relationship between TILs and sex of RCC patients.*

TILs did not show dependence on age, histological variant, vascular invasion, N and M-stage and localization of RCC.

Vascular invasion did not correlate with the following clinical-morphological indicators: age, sex, histological variant, spread (N and M-stage) and localization of RCC.

4.2.4. Analysis of differentiation degree in relation to clinical-morphological indicators

Relationship between differentiation degree and histological variant of RCC

Table 7 presents data on the distribution of patients using the four degrees of differentiation by the ISUP system according to the histological variant of RCC. Comparative analysis showed a statistically significant relationship between the indicators (χ^2=8.634, p=0,035). It is noteworthy that in pRCC there were no tumors with G1 and G4 differentiation, while in ccRCC there were more tumors with low degree of differentiation (G3 and G4).

Our data differ from those of Bretheau et al. (1995), who found no relationship between the degree of differentiation determined

by the Fuhrman system and the histological variant of the tumor. The difference between our study and that of Bretheau et al. (1995) may be due to selection criteria in our study and/or different differentiation systems used.

Table 7. *Distribution of RCC cases by histological variant and differentiation degree.*

Group	G1	G2	G3	G4	Total
pRCC	0 (0,0%)	14 (70,0%)	6 (30,0%)	0 (0,0%)	20 (100%)
ccRCC	3 (7,7%)	14 (35,9%)	15 (38,5%)	7 (17,9%)	39 (100%)
Total	3 (5,1%)	28 (47,4%)	21 (35,6%)	7 (11,9%)	59 (100%)

Relationship between differentiation degree and vascular invasion in RCC

A statistically significant difference was found between ccRCC and pRCC differentiation degree and LVI (χ^2=14.130, p=0,003) (Table 8). As the tumor differentiation degree decreased, the probability of vascular invasion increased.

Table 8*. Distribution of RCC cases by LVI and differentiation degre*e.

Group	G1	G2	G3	G4	Total
absence of LVI	3 (9,1%)	21 (63,6%)	8 (24,2%)	1 (3%)	33 (100%)
presence of LVI	0 (0,0%)	7 (26,9%)	13 (50,0%)	6 (23,1%)	26 (100%)
Total	3 (5,1%)	28 (47,5%)	21 (35,6%)	7 (11,8%)	59 (100%)

Bedke et al. (2018) reported results similar to ours. They examined MVI and LVI in 747 RCC cases, finding invasion in 201 of them. The authors found a significant relationship between MVI and LVI and differentiation degree determined by the Fuhrman system,

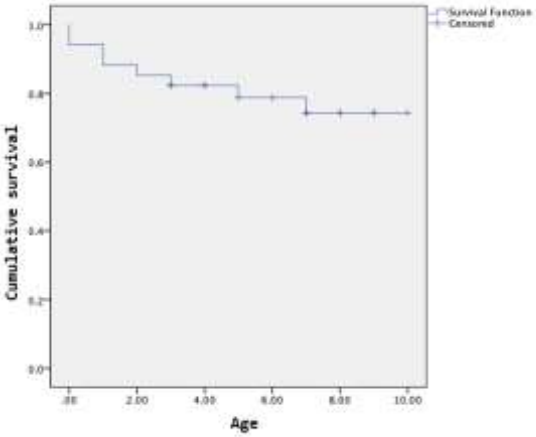
with invasion associated with lower tumor differentiation (Bedke J et al., 2018). According to Bedke et al. (2018), MVI and LVI are poor prognostic factors in renal carcinoma.

RCC differentiation degree did not correlate with patient age and sex, tumor necrosis area, TILs, N and M-stage and tumor localization.

4.3. Survival of patients with renal cell carcinoma

Of the 80 patients studied, survival information was available for 34, of whom 26 were alive as of January 2022 and 8 had died. The mean survival for the follow-up period of these patients was 8.1 years (95% CI 6.96-9.30). Figure 10 shows the Kaplan Meier survival curve for RCC patients.

Figure 10. *Cumulative survival curve of RCC patients.*



We analyzed the survival of RCC patients in relation to various clinical-morphological indicators.

Relationship between survival and age of RCC patients

Examining survival in relation to the age of the studied patients, a weak inverse relationship (r= - 0,352, p=0,041) was found between the two variables. The data indicated that the older the patients, the lower the probability of survival by 2022 (Fig. 11).

According to other authors (Feng X et al., 2019), overall survival of ccRCC patients decreased significantly with advancing age at diagnosis. Similar results were obtained by other authors in ccRCC (Liao Z et al., 2022).

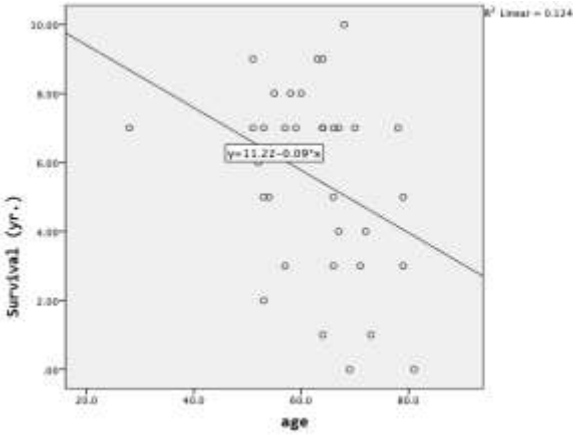


Figure 11. *Correlation between survival and age of RCC patients.*

Relationship between survival and sex of RCC patients

We found a difference in survival between the two sexes (p=0,044) (Fig. 12). The mean survival for men was 8.7 years (95% CI from 7.43 to 9.89), 5.1 years for women (95% CI from 3.15 to - 7.10). The data indicated that survival in men was higher than in women.

Our results differed from data obtained in other studies. Chang et al. (2011) reported that in the 328 RCC cases studied, five-year

overall survival for women was 79.5%, and for men 73.7%. The reasons for these differences may be related to the different number of patients included in the two studies and/or the different duration of follow-up. Feng et al. (2019) also report that in ccRCC, overall survival in women was significantly higher than in men (median survival for women: 156 months, men: 132 months).

The results from our study also differ from those by Spasova (2018). She analyzed the survival of patients with metastatic RCC and sex and found no statistically significant relationship between the two indicators: patient sex and months of survival.

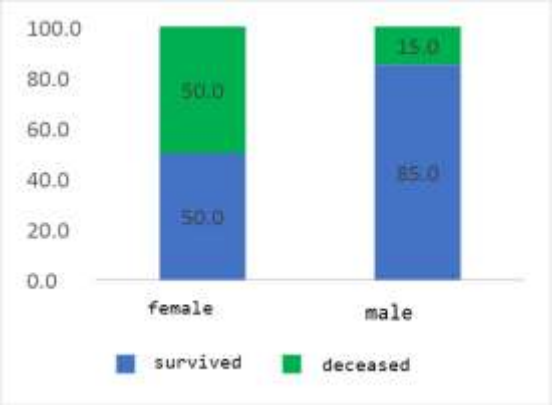


Figure 12. *Survival of RCC patients in relation to sex.*

Relationship between survival and extent of tumor necrosis spread

In analyzing the survival of RCC patients in relation to the extent of TN spread, we found a statistically significant relationship (F=4.815, p=0,007). The mean survival of patients varies depending on the spread of necrosis. The larger the area of necrosis, the lower the survival of patients (Fig. 13).

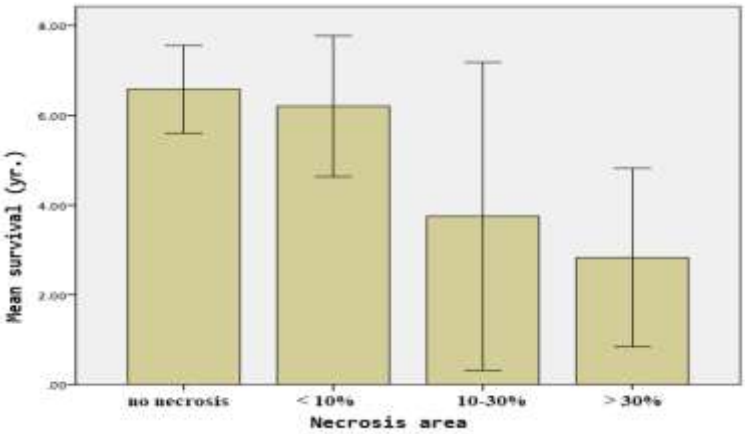


Figure 13. *Mean survival of RCC patients in relation to TN and its spread area.*

According to Sengupta et al. (2005), the presence of coagulative TN has different prognostic significance in ccRCC, pRCC and . In patients with ccRCC and chRCC, 10-year cancer-specific survival was 77.6% and 90.0%, respectively, in the absence of TN, but only 29.2% and 68.3% when present. Despite the higher prevalence of coagulative TN in pRCC and its association with some unfavorable morphological features, it had no prognostic significance. 10-year tumor-specific survival was 85.4% and 88.9% for patients with and without necrosis (Sengupta S et al., 2005).

In metastatic RCC, Spasova (2018) reported the highest mean overall survival in patients without tumor necrosis (46.06 months). The mean values decreased with the appearance of necrosis and area increase. Despite this trend, Spasova (2018) found no statistically significant relationship between tumor necrosis and patient mortality.

In their study, Lam J et al. (2005) found that patients with necrosis in the tumor tissue of primary RCC had lower 5-year disease-specific survival compared to patients without necrosis in the primary tumor (36% versus 75%). Significantly lower 5-year disease-free

survival (62% versus 92%) was found in patients with necrosis compared to patients without necrosis in the primary tumor in localized RCC.

Relationship between survival and vascular invasion in RCC

We found a statistically significant relationship between survival and LVI (p=0,001) in the 34 patients studied. Survival of patients without LVI was higher (Fig. 14).

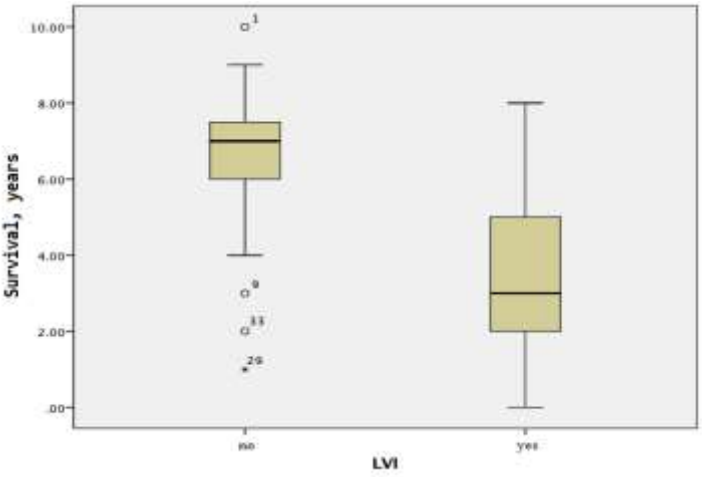


Figure 14. *Survival of RCC patients in relation to LVI.*

Bedke et al. (2018) also found a significant relationship between MVI and cancer-specific survival in ccRCC patients. They established that microvascular, not macrovascular, invasion was an independent predictive factor for metastatic tumor spread. According to other authors, MVI was an independent predictive indicator for disease recurrence and was the most important factor associated with fatal outcome in RCC patients (Dall'Oglio MF et al., 2007).

Relationship between survival and T stage of RCC

We found a statistically significant relationship between the risk of mortality during the follow-up period and the T stage of the 34 patients examined. As the stage increased, so did the risk of mortality (χ^2=11.161, p=0,011) (Fig. 15).

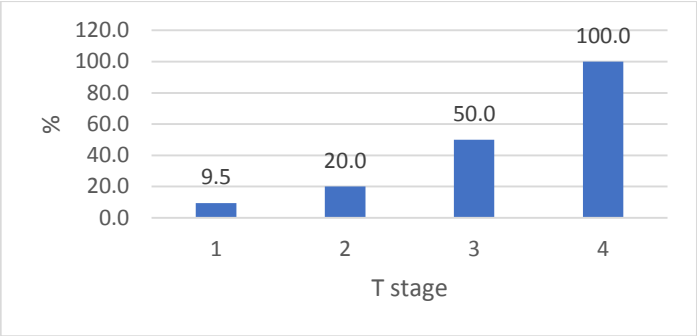


Figure 15. *Relationship between the risk of mortality and T stage of RCC.*

There is literature data on the significant association between these two indicators. In their study on RCC, Chang et al. (2011) monitored the patients over an average period of 46.5 months. According to their data, survival was 82.3% at stage T1, 84.5% at T2, 57.5% at T3, and 0.0% at T4. The authors suggest that the TNM stage and T stage were the most important prognostic factors for the overall survival of RCC patients. Liao et al. (2022) observed a relationship between cancer-specific survival and overall survival with the TNM stage in ccRCC.

In her study, Spasova (2018) performed a comparative analysis of T stage and overall survival in patients with metastatic

RCC. She demonstrated that tumor size was of definitive prognostic importance regarding overall survival, finding a significant difference in survival based on tumor size. According to the data, the highest number of deaths occurred in patients with metastatic RCC diagnosed at T3 (15 cases, 50%). The highest overall survival (20.66±15.52 months) was observed in T2 stage patients, which was surprisingly greater than in T1 stage patients (18.50±12.46). The lowest overall survival was in T4 patients (10.00±9.59 months), while for T3 it was 14.80±12.64 months (Spasova, 2018).

Survival in RCC patients did not correlate with histological variant, degree of differentiation, TILs, or carcinoma location.

4.4 Expression of AIF in RCC tumor tissue, adjacent non-tumor tissue, and distant metastases

Expression of AIF in tumor tissue in RCC

Cytoplasmic and nuclear expression (Fig. 16A and Fig. 16B) of AIF was studied in the tumor tissue of all 80 patients. The mean cytoplasmic expression determined by H-score was 168,3 (SD=36,88), with a minimum value of 95 and a maximum of 250. The mean nuclear expression of AIF was 2,1 (SD=10,57), with a minimum value of 0 and a maximum of 85.

The values obtained in this study differ from other data published in the literature (Jeong EG et al., 2006; Krasnik V et al., 2017). The reasons may be tissue-dependent and/or due to the different methods used to assess AIF expression.

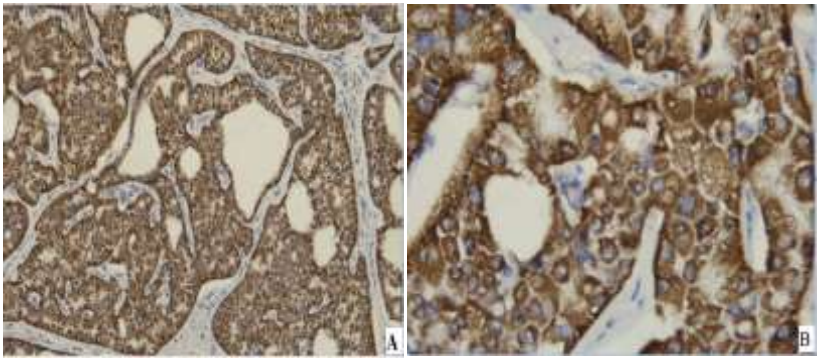


***Figure 16**. A/ High cytoplasmic and absent nuclear expression of AIF in RCC x100; B/ High cytoplasmic and absent nuclear expression of AIF in RCC x400.*

In the study by Krasnik et al. (2017), which included 54 patients with uveal melanoma, cytoplasmic AIF expression was determined as the percentage of positive cells on a scale from 1 to 6 (1 = 1-4%; 2 = 5-19%; 3 = 20-39%; 4 = 40-59%; 5 = 60-79%; and 6 = 80-100%). The intensity of positive cell staining was assessed from 0 to 3 (0 = no staining; 1 = weak; 2 = moderate; and 3 = strong staining). The multiplicative score was obtained by multiplying the percentage of positive cells by the staining intensity, with the resulting score ranging from 0 to a maximum of 18. Thirty-three (67%) of the patients had a score ≥ 4. No nuclear expression was observed.

In their study, Jeong et al. (2006) assessed cytoplasmic AIF expression in CRC cells. A positive reaction was found in all 103 patients. The authors applied a three-point scale for evaluation: +, ++, and +++, finding that the intensity was mildly expressed (+) in 20 cases, moderately expressed (++) in 30 cases, and strongly expressed (+++) in 53 cases.

Expression of AIF in adjacent non-tumor tissue

In 40 patients in the present study, cytoplasmic AIF expression was assessed in the epithelial cells of the renal tubules in adjacent non-tumor tissue (Fig. 17). The mean cytoplasmic AIF expression in non-tumor tissue was 172,7 (SD=46,14), with a minimum of 100 and a maximum of 280, while the mean cytoplasmic expression in the tumor tissue of these 40 patients was 168,8 (SD=38,03).

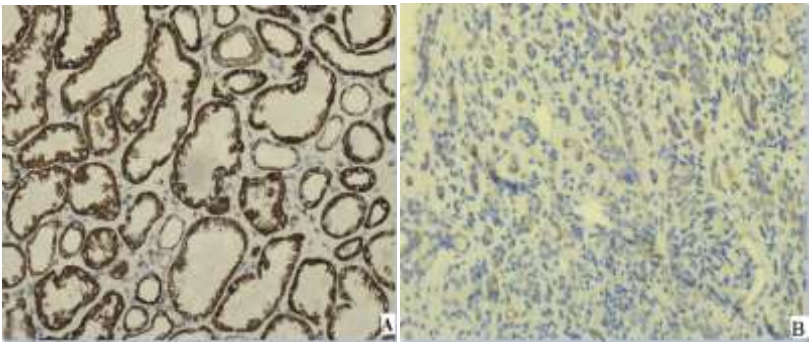


Figure 17. *A/ High cytoplasmic expression of AIF in adjacent non-tumor tissue x200; B/ Low cytoplasmic expression of AIF in adjacent non-tumor tissue x100.*

The mean expression values in normal and tumor tissue in this study were approximately the same. Our results differed from other published data (Wang Z et al., 2019). Wang et al. (2019) studied AIF expression in tumor and adjacent non-tumor tissue in 96 RCC cases. The authors determined an overall score as the sum of the average percentage of positive cells on the following scale: ≤5% - 0 points; 6–25% - 1 point; 26–50% - 2 points; 51–75% - 3 points; and >75% - 4 points, with the intensity of the reaction graded in four categories: from no staining (0) to dark brown (4 points). The total score was classified as negative (0–2), weakly positive (3–5), or strongly

positive (6–8). In tumor tissue, AIF values were significantly lower compared to normal renal tissue, which showed an intense reaction (Wang Z et al., 2019).

Expression of AIF in distant metastases

Of the 80 patients in the present study, 15 had histologically verified distant metastases (pM1) with various localizations. We determined cytoplasmic AIF expression (Fig. 18) in 14 of the metastases and obtained a mean value of 147,5 (SD=46,51), with a minimum of 65 and a maximum of 215. The mean AIF value in primary RCC was 151,8, with a minimum of 95 and a maximum of 225.

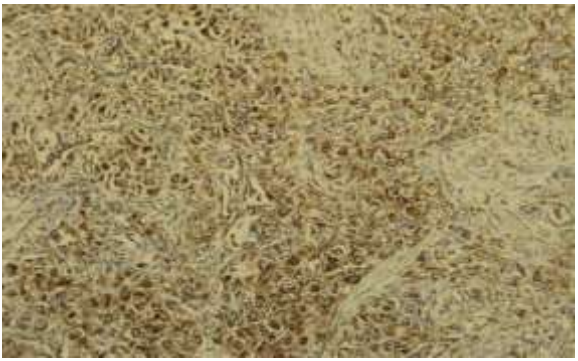


Figure 18*. AIF expression in the cytoplasm of tumor cells in RCC lung metastasis x200.*

4.4.1. Comparative analysis of AIF expression in tumor tissue and adjacent non-tumor tissue

When analyzing the mean cytoplasmic expression values of the apoptosis-inducing antibody in tumor and non-tumor cells (Table 9), no statistically significant relationship was found (p=0,627). Our

results showed that apoptosis in RCC tumor tissue, which can be induced by AIF, was not suppressed.

Wang et al. (2019) determined AIF in RCC immunohistochemically and by RT-qPCR, finding that the apoptosis-inducing factor was significantly lower in tumor tissue compared to normal tissue. According to the authors, reduced AIF expression was associated with renal tumorigenesis. The overall survival of patients, followed up postoperatively for 6 to 118 months, showed that negative expression was associated with lower survival compared to patients with positive expression (Wang Z et al., 2019).

***Table 9**. Mean cytoplasmic AIF expression in RCC tumor tissue and adjacent non-tumor tissue.*

Group	Number of cases (n)	Mean cytoplasmic AIF expression	Standard deviation (SD)	P-value
In tumor cells	40	172,7	46,14	**0,627**
In non-tumor cells	40	168,8	38,03	

In addition to RCC, low AIF expression was also found in other tumors. Letkovska et al. (2021) examined cytoplasmic AIF expression in 216 testicular tumors, reporting that expression in spermatogenic cells of non-tumor testicular tissue was significantly higher compared to that in tumor cells.

Increased AIF expression was also observed in tumor tissue of other tumors. Lee et al. (2006) studied cytoplasmic AIF expression in tumor and non-tumor tissue in 60 cases of gastric carcinoma. They used a three-point scale to assess immunohistochemical reaction: -, +,

and ++. Positive expression was found in 42 cases, while in normal gastric mucosa, positivity was observed only in parietal cells. Stromal cells, fibroblasts, and smooth muscle cells were negative. According to Lee et al. (2006), increased AIF expression did not depend on histological type or the depth of invasion of gastric carcinoma. The same authors suggest that increased expression of the apoptosis marker in tumor cells compared to normal mucosal cells indicated that AIF expression might play a role in gastric tumorigenesis (Lee JW et al., 2006).

4.4.2. Comparative analysis between cytoplasmic and nuclear expression of AIF in tumor tissue

When analyzing mean values of cytoplasmic and nuclear expression of AIF, we observed a statistically significant difference, with cytoplasmic expression being significantly higher compared to nuclear expression of the antibody in tumor cells of RCC ($p < 0,001$) (Fig. 19).

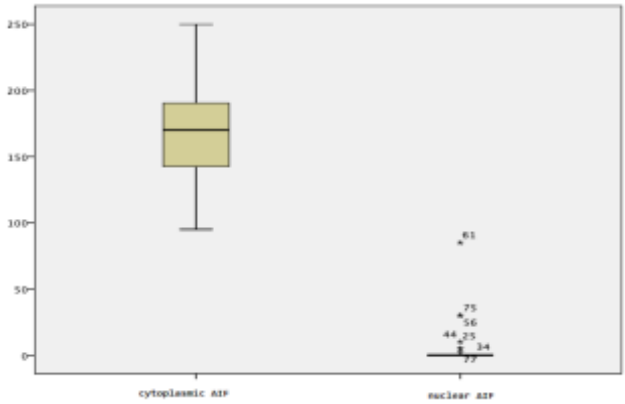


Figure 19. *Relationship between cytoplasmic and nuclear expression of AIF in tumor cells of RCC.*

4.4.3. Comparative analysis between cytoplasmic expression of AIF in primary tumor and distant metastases

In the comparative analysis of cytoplasmic expression of AIF in the primary tumor and in distant metastases, no significant difference was found between the mean values (p = 0,737) (Table 10).

Table 10. *Mean values of cytoplasmic expression of AIF in tumor cells of primary RCC and distant metastases.*

Group	Number of cases (n)	Mean cytoplasmic expression of AIF	Standard Deviation (SD)	p-value
AIF primary tumor	14	151,8	36,43	**0,737**
AIF metastasis	14	147,5	46,51	

Similar results, where no relationship was found between AIF expression in the primary tumor and distant metastases, were observed in other tumors such as CRC and gastric carcinoma (Jeong EG et al., 2006; Lee JW et al., 2006). Letkovska et al. (2021) analyzed AIF expression in tumor tissue of germ cell testicular tumors and found reduced AIF expression compared to non-tumorous tissue. Patients with more advanced disease (those with three or more metastases, mediastinal lymph node metastases, liver metastases, or other non-pulmonary visceral metastases) had lower levels of AIF in tumor tissue compared to patients with better/intermediate prognosis and less advanced disease. However, these differences were statistically insignificant, except for patients with non-pulmonary visceral metastases, who had lower AIF expression compared to those without such metastases (Letkovska K et al., 2021). According to the authors, the reduction of AIF might represent one of the mechanisms of

apoptosis inhibition, facilitating cell survival and metastasis (Letkovska K et al., 2021).

4.5. Comparative analysis of cytoplasmic expression of AIF in RCC in relation to clinicopathological indicators and patient survival

Relationship between cytoplasmic expression of AIF and patient age

There was no statistical relationship between the parameters of cytoplasmic expression of AIF and patient age (F=2,513, p= 0,088). Upon retesting the two parameters using Spearman's correlation method, a weak but significant relationship was observed (ρ=0,23, p=0,039) (Fig. 20). The values of AIF increased with advancing age.

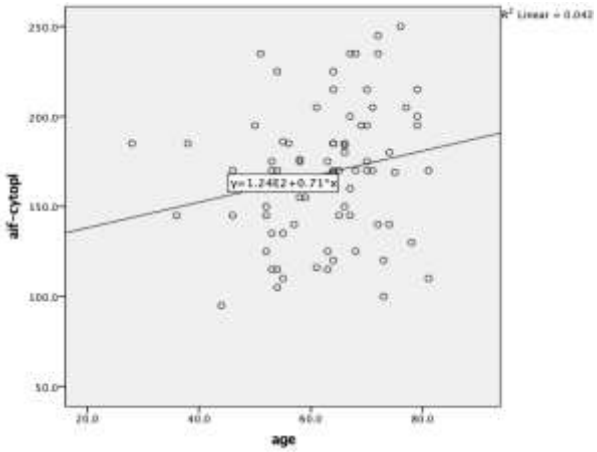


Figure 20. *Relationship between cytoplasmic expression of AIF in tumor tissue of RCC and patient age.*

A lack of correlation between cytoplasmic AIF expression and patient age has been observed not only in ccRCC (Xu S et al., 2014), but also in CRC (Jeong EG et al., 2006) and gastric cancer (Lee JW et al., 2006). However, some authors found a relationship between these two parameters in RCC. Our results differ from those of Wang Z et al. (2019) for RCC, where the frequency of positive AIF expression in patients aged <60 years was higher than that in patients aged ≥60 years.

Correlation between cytoplasmic AIF expression and tumor histological variant

We analyzed cytoplasmic AIF expression in relation to the histological variant of RCC using analysis of variance. A significant correlation was found between only two of the RCC variants: pRCC and ccRCC (p=0,003). The mean expression value in the pRCC group was 185,0 and was higher than that in the clear cell group, which was 154,6. Table 11 shows the mean cytoplasmic expression values of AIF according to the histological variant.

Table 11. *Mean values of cytoplasmic AIF expression according to the histological variant of RCC.*

Histological Variant	Number of cases (n)	Mean cytoplasmic expression of AIF	Standard Deviation (SD)
pRCC	20	185,0	42,17
chRCC	21	177,8	25,15
ccRCC	39	154,6	34,90

In gastric carcinoma, cytoplasmic AIF expression in tumor cells did not show a relationship with histological type (intestinal and diffuse) (Lee JW et al., 2006). In germ cell testicular tumors,

Letkovska et al. (2021) reported that AIF expression was significantly higher in non-seminoma tumors compared to seminomas.

Relationship between cytoplasmic AIF expression and vascular invasion

Analyzing AIF cytoplasmic expression in tumor cells in relation to LVI, we found a statistically significant correlation (p=0,014). Cytoplasmic expression values were higher when tumor emboli were absent, and lower when they were present (Table 12).

Table 12. *Correlation between mean cytoplasmic expression of AIF and LVI in RCC.*

Vascular Invasion	Number of Cases (n)	Mean Cytoplasmic Expression of AIF	Standard Deviation (SD)	p-value
present	33	156,4	39,33	0,014
absent	47	176,7	32,96	

Relationship between cytoplasmic AIF expression and T stage of RCC

AIF expression in the cytoplasm of tumor cells was analyzed in relation to different T stages by analysis of variance. We found a statistically significant difference between two of the T stages: T1 and T3 (p=0,001) (Table 13). In smaller tumor size, the expression of the apoptosis marker was higher in contrast to tumors of larger size where the expression decreased.

Another study on AIF expression in RCC also found a statistically significant difference in relation to T stage (Wang Z et al., 2019). The authors reported that in terms of clinical T stage, AIF-

positive expression was significantly lower in stage T3 carcinoma than in stage T1. In another study including only ccRCC, no association was found between AIF expression and tumor size (Xu S et al., 2014). The reasons for these differences are unknown.

Table 13. *Mean values of cytoplasmic AIF expression in tumor cells by T stage of RCC.*

T stage	Number of cases (n)	Mean cytoplasmic expression of AIF	Standard deviation (SD)
T1	35	184,6	31,97
T2	14	169,9	30,44
T3	26	147,7	37,44
T4	5	157,0	34,02

We found no statistically significant relationship between cytoplasmic AIF expression and the following clinicopathological factors: patient sex, tumor differentiation grade, extent of tumor necrosis, TILs (tumor-infiltrating lymphocytes), N and M stage, tumor location or patient survival with RCC.

4.6. Comparative analysis of nuclear AIF expression in relation to clinical-morphological characteristics of the tumor and survival of RCC patients

Nuclear expression of AIF in renal cell carcinoma did not correlate with any of the investigated clinical-morphological parameters: age, sex, histological variant, degree of carcinoma differentiation, tumor necrosis, TILs, LVI, T, N and M stage, tumor localization and patient survival.

In the literature available to us, not much data was found evaluating immunohistochemical nuclear expression of AIF in

relation to clinical-morphological parameters in RCC. With a focus on nuclear expression of AIF is the study by Krasnik et al. (2017) on uveal melanoma and the relationship of nuclear expression to patient prognosis. The authors found no such localization of AIF, although it was consistently present in the cytoplasm of tumor cells.

The subcellular localization of AIF in RCC was studied by Wang et al. (2019) using immunofluorescence. In non-tumor tissue, AIF was more often present in mitochondria than in the nucleus. In tumor tissue, nuclear expression increased while mitochondrial expression decreased, and these changes were associated with the degree of differentiation of RCC. The highest nuclear expression was in G4 of the tumor and lowest in G1. Conversely, mitochondrial expression was highest in G1 and decreased with lower degrees of differentiation (Wang Z et al., 2019). According to the authors, these results suggested that AIF underwent nuclear translocation in RCC tissues, depending on the degree of tumor differentiation (Wang Z et al., 2019).

4.7. Expression of RIPK3 in RCC tumor tissue, adjacent non-tumor epithelial tissue and distant metastases

Cytoplasmic (Fig. 21) and nuclear expression of RIPK3 (Fig. 22) was examined in tumor tissue of all 80 patients. The mean value of cytoplasmic expression of RIPK3, determined by H-score, was 137,1 (SD=43,96) with a minimum value of 0 and a maximum of 285. The mean value of nuclear expression of RIPK3 was 27,9 (SD=46,89) with a minimum value of 0 and a maximum of 195.

The mean value of cytoplasmic expression in the present study differed from the mean value of cytoplasmic expression of RIPK3 in breast carcinoma (119.6±56.4), also determined by H-score (Stoeva M., 2022). There was a substantial difference in terms of the

mean value of nuclear expression in the present study and the study by Stoeva (2022), where the value was 189.4±54.2.

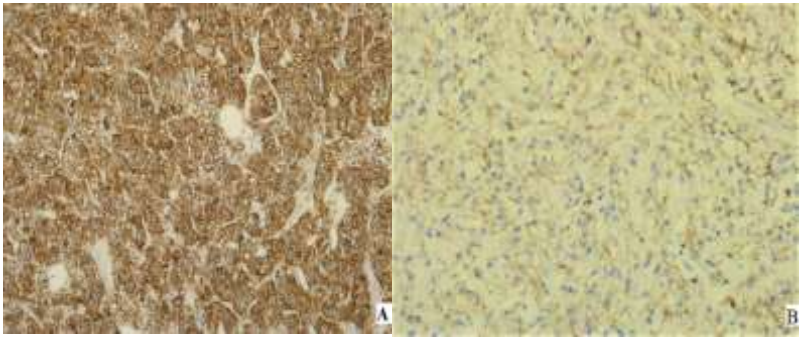


Figure 21. *A/ Intense cytoplasmic expression of RIPK3 in RCC cells, x100; B/ Weak cytoplasmic expression of RIPK3 in RCC cells, x200.*

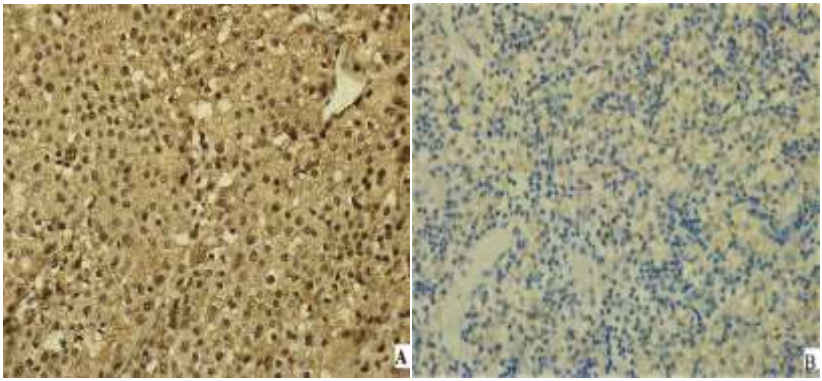


Figure 22. *A/ Moderate to intense nuclear expression of RIPK3 in RCC cells, x 200; B/ Weak to absent nuclear expression of RIPK3 in RCC cells, x 200.*

In 40 of the RCC cases, cytoplasmic expression was also determined in epithelial cells of renal tubules in adjacent non-tumor tissue (Fig. 23). The mean value of cytoplasmic expression of RIPK3 in non-tumor tissue was 171,75 (SD=41,84) with a minimum of 75 and a maximum of 270, while the mean value of cytoplasmic expression in tumor tissue in these 40 patients was 149,8 (SD=45,59) (Table 14).

In CRC (Stefanova N, 2017), analysis of cytoplasmic expression of RIPK3 in adjacent non-tumor tissue, based on mean values, showed lower levels of expression compared to ours (149.38±92.00).

Stoeva (2022) studied the cytoplasmic expression of RIPK3 in 19 cases of fibrocystic disease, divided into two groups: non-proliferative and proliferative type. The mean value in the proliferative type fibrocystic disease group was 180.6±8.4, and 187.5±10.1 in the non-proliferative type. Mean values of cytoplasmic expression of RIPK3 in this mammary carcinoma did not differ substantially from those in the present study (Stoeva M, 2022).

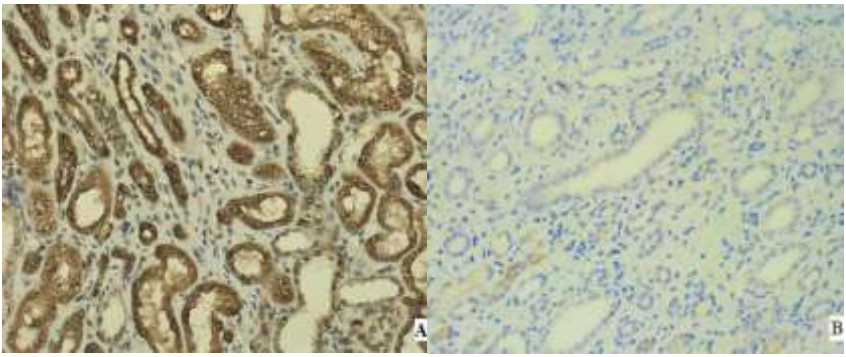


Figure 23. *A/ Moderate to intense cytoplasmic expression of RIPK3 in adjacent non-tumor tissue x200; B/ Weak to absent cytoplasmic expression of RIPK3 in adjacent non-tumor tissue x200.*

Table 14. *Mean values of cytoplasmic expression of RIPK3 in tumor and non-tumor epithelial cells in RCC.*

Group	Number of cases (n)	Mean cytoplasmic expression of RIPK3	Standard deviation (SD)
tumor cells	40	149,8	45,59
non-tumor epithelial cells	40	171,7	41,84

In 15 of the studied patients, there were histologically verified distant metastases (pM1) with different localizations. We determined the cytoplasmic expression (Fig. 24) of RIPK3 in 14 of the metastases and found that the mean value was 130,6 (SD=24,71) (Fig. 25), while in primary RCCs it was 143,7 (SD=33,14) (Fig. 26).

Figure 24. *Cytoplasmic expression of RIPK3 in tumor cells from RCC lung metastasis, x100.*

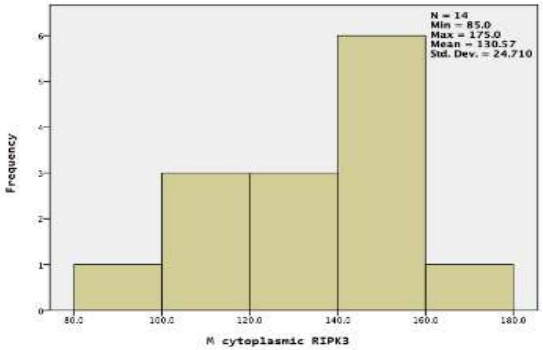


Figure 25. *Cytoplasmic expression of RIPK3 in tumor tissue of RCC metastases.*

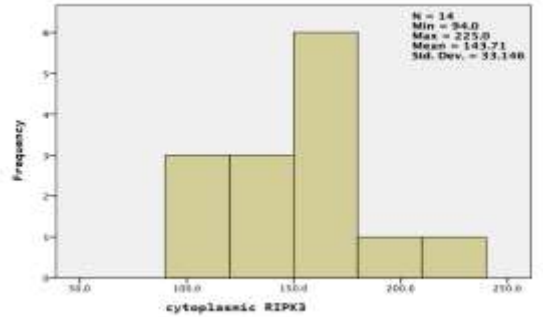


Figure 26. *Cytoplasmic expression of RIPK3 in RCC tumor tissue with distant metastases.*

4.7.1. Comparative analysis between mean values of RIPK3 expression in tumor tissue and adjacent non-tumor tissue.

When comparing the values of cytoplasmic expression of RIPK3 in tumor and adjacent non-tumor tissue in the 40 cases studied, we found a significant statistical difference (p=0,017) (Fig. 27). Cytoplasmic expression in tumor tissue was lower than that in adjacent non-tumor tissue.

Our results did not differ from those of Feng et al. (2015), who evaluated immunohistochemical cytoplasmic expression of RIPK3 in tumor tissue in patients with colorectal carcinoma and compared it with that in normal mucosa, using a three-step intensity rating scale: 0, 1 and 2. They found higher cytoplasmic levels of RIPK3 expression in normal mucosa compared to tumor tissue. Similar results of decreased tissue expression levels of RIPK3 in CRC, compared to adjacent non-tumor tissue, were also found by Moriwaki et al. (2015). Our results did not differ from those of Stoeva (2022), who examined cytoplasmic expression of RIPK3 in tumor tissue of breast carcinoma patients and in fibrocystic disease controls. Expression in controls was higher.

Regarding CRC, Stefanova (2017) did not find a statistically significant difference in cytoplasmic positivity of RIPK3 between tumor and non-tumor tissue.

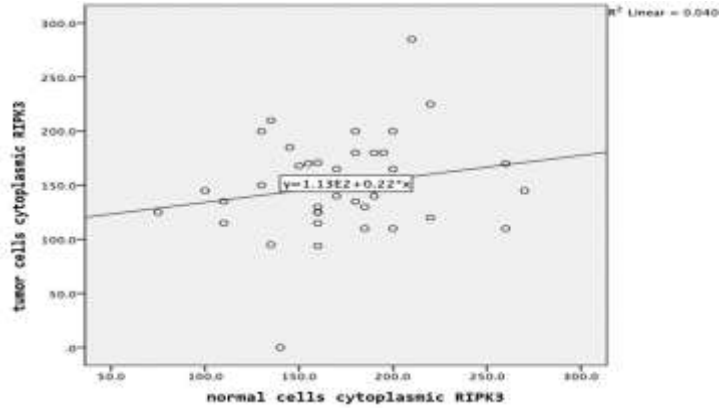


Figure 27. *Correlation between cytoplasmic expression of RIPK3 in tumor cells and in normal renal tubular cells.*

4.7.2. Comparative analysis between cytoplasmic and nuclear expression of RIPK3 in tumor tissue

Figure 28 shows the mean values of cytoplasmic and nuclear expression of RIPK3 in RCC tumor cells. The difference between

them was statistically significant (p<0,001), with mean values of cytoplasmic expression higher than those of nuclear expression.

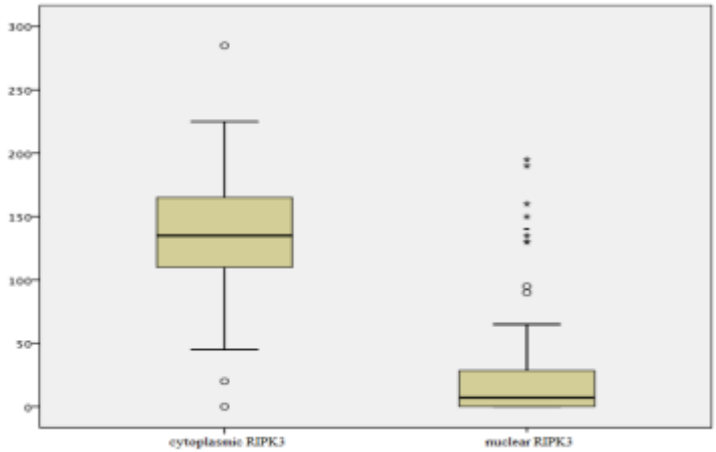


Figure 28. *Mean values of cytoplasmic and nuclear expression of RIPK3 in RCC tumor cells.*

In her study, Stoeva (2022) analyzed the relationship between nuclear and cytoplasmic expression of RIPK3 in tumor tissue of mammary carcinoma and, unlike us, did not find a significant relationship between the two indicators. In our opinion, these differences might be tissue-specific.

4.7.3. Comparative analysis between cytoplasmic expression of RIPK3 in RCC tumor tissue and distant metastases

Figure 29 presents the mean values of cytoplasmic expression of RIPK3 in tumor tissue of 14 of the metastases and primary RCC. No significant difference was found between the two values (p= 0,191).

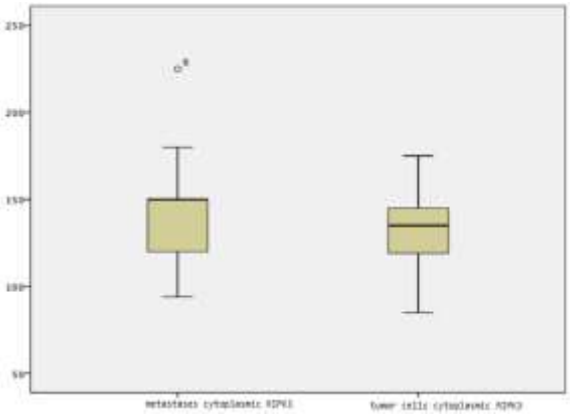


Figure 29. *Mean values of RIPK3 cytoplasmic expression in RCC tumor tissue and distant metastases.*

4.8. Comparative analysis of cytoplasmic expression of RIPK3 according to clinical-morphological characteristics and patient survival

Relationship between cytoplasmic expression of RIPK3 and patient age

Figure 30 shows the mean values of cytoplasmic expression of RIPK3 relative to patient age. No significant relationship was found between the two indicators (p=0,081). Upon retesting cytoplasmic expression of RIPK3 in tumor cells in relation to patient age using the Spearman method, a weak significant relationship was found (ρ=0,222, p=0,048). Expression of RIPK3 in RCC tumor tissue increased with advancing patient age.

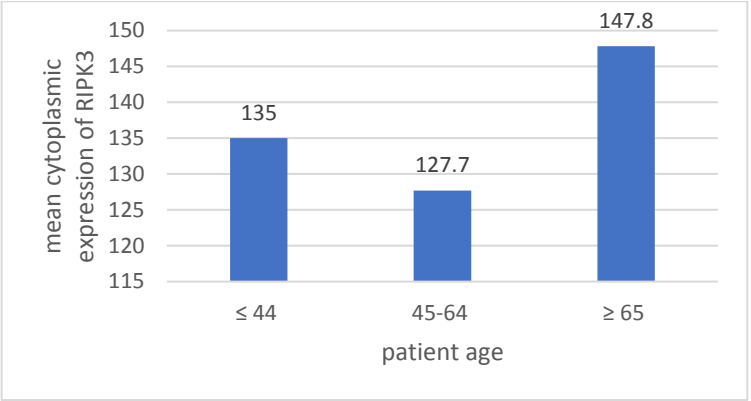


Figure 30. *Mean cytoplasmic expression of RIPK3 in RCC and patient age.*

In the study by Feng et al. (2015), no relationship was found between cytoplasmic expression of RIPK3 in the tumor and patient age in CRC. Patients were divided into two age groups: <65 and ≥65 years. Similar results were obtained by Stoeva (2022) when comparing the two indicators in breast carcinoma. Chung et al. (2019) did not find a relationship between expression of RIPK3 in tumor cell cytoplasm and age in patients with lung adenocarcinoma who underwent cisplatin-based chemotherapy after lung resection.

Relationship between cytoplasmic expression of RIPK3 and degree of RCC differentiation

Figure 31 shows the mean values of cytoplasmic expression of RIPK3 in papillary and clear cell RCC with different degrees of differentiation (G1-G4) determined by the ISUP system. A statistically significant relationship was found between the indicators (F=3,314, p=0,026), with mean values of RIPK3 in the G2 differentiation grade group higher than G4.

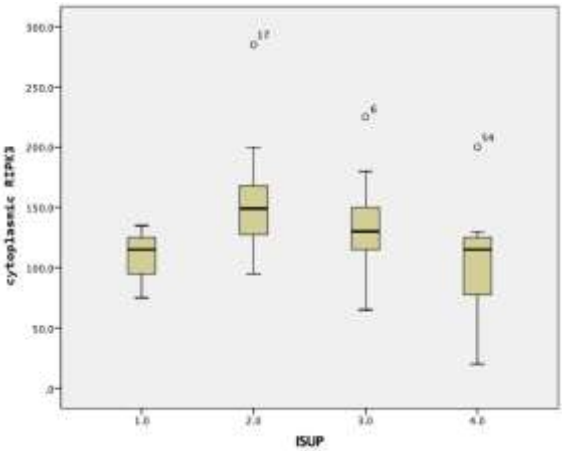


Figure 31. *Mean cytoplasmic expression of RIPK3 in the two RCCs: pRCC and ccRCC, with different degrees of differentiation (G1-G4) determined by the ISUP system.*

There is data in literature indicating no statistically significant relationship between cytoplasmic levels of RIPK3 and degree of differentiation in CRC (Feng et al. 2015; Stefanova N, 2017). The results from Stoeva's study (2022) on breast carcinoma were similar to those obtained by us and showed a dependence of RIPK3 expression in tumor cells on the degree of differentiation. High expression was associated with better tumor differentiation.

Relationship between cytoplasmic expression of RIPK3 and vascular invasion

Analyzing the expression of RIPK3 in tumor cell cytoplasm in relation to LVI, we found a statistically significant relationship (p=0,031). Table 15 shows the mean values of the two indicators.

Mean values of RIPK3 were higher in the presence of vascular invasion.

Table 15. *Relationship between mean cytoplasmic expression of RIPK3 and LVI in RCC.*

vascular invasion	number of cases (n)	mean values of cytoplasmic RIPK3	Standard deviation (SD)	P-value
present	33	149,7	45,72	0,031
absent	47	128,3	40,87	

Our data differ from those in CRC. Analyzing cytoplasmic expression of RIPK3 and vascular invasion, Stefanova (2017) did not find a statistically significant difference. Similar results have been published by Feng et al. (2015), who also did not find statistical significance between the two indicators. The differences between literature data and the present study might be tissue-specific.

Cytoplasmic expression of RIPK3 in RCC tumor cells showed no correlation with the following clinical-morphological indicators: patient sex, histological variant, TN area, TILs, T, N and M stage, tumor localization and patient survival.

4.9. Comparative analysis of nuclear expression of RIPK3 according to clinical-morphological indicators and patient survival

Nuclear expression of RIPK3 in renal cell carcinoma tumor tissue did not correlate with any of the investigated clinical-morphological indicators: patient age and sex, histological variant, degree of differentiation, necrosis area, TILs, vascular invasion, T, N and M stage, tumor localization and patient survival.

In her study on CRC, Stefanova (2017) also did not find a significant relationship between nuclear expression of RIPK3 and patient age and sex, degree of differentiation, TN area, LVI, T- and N-stage and tumor localization.

Stoeva (2022), in her study on breast carcinoma, also did not find a relationship between nuclear expression of the necroptosis

marker and patient age, histological variant and degree of carcinoma differentiation, T- and M-stage.

Taken together, our results showed that nuclear translocation of RIPK3 was not related to the clinical-morphological indicators of RCC.

V. SUMMARY

RCC incidence and mortality have shown a constant tendency to increase worldwide over the years. Patient diagnosis is often made at an advanced stage of the carcinoma due to the late onset of clinical symptoms. New information is constantly emerging about the diversity of genetic characteristics and features of these tumors, which needs to be taken into account in their histological verification and treatment. Currently known standard approaches for RCC treatment are insufficient in some cases due to resistance to chemo- and radiotherapy. There is a growing need for new and different treatment strategies for cases that do not respond to classical therapy, as well as in cases of regional and distant tumor spread.

Pathway activation of one or another form of cell death can be used in the search for new approaches in RCC treatment. There is evidence in the literature that renal carcinoma tumor cells are resistant to the intrinsic and extrinsic pathways of apoptosis. Apoptosis is programmed cell death that can also occur via another, alternative pathway that is caspase-independent and involves AIF. The role of AIF in RCC is poorly studied and additional research is needed, which is why it was the object of the present study.

Another form of cell death is necroptosis. A key participant in the process is RIPK3, which is known to have both tumor-stimulating and tumor-suppressive functions in different tumors. Further in-depth studies are needed on the role of RIPK3 in tumor development,

progression, metastases, recurrence, as well as anti-tumor immunity in RCC, and also its correlation with clinical-morphological indicators.

VI. CONCLUSIONS

1. In advanced renal cell carcinoma (T3 and T4 stage), tumors showed a lower degree of differentiation, larger necrosis area, higher TILs intensity, and had a higher risk of death compared to earlier stages of carcinoma (T1 and T2 stage).
2. T stage of renal cell carcinoma showed no dependence on age, sex, histological variant, N and M stage, and tumor localization.
3. Tumor necrosis was more common in papillary renal cell carcinoma than in its chromophobe variant. Of the clinical-morphological indicators, large tumor necrosis area correlated with lymphovascular invasion, right-sided tumor localization, and low patient survival.
4. In renal cell carcinoma, TILs were more often absent in females compared to males.
5. In the absence of vascular invasion, survival of patients with renal cell carcinoma was higher compared to patients where it was present.
6. In clear cell renal cell carcinoma, low degrees of tumor differentiation (G3 and G4) predominated, while in papillary carcinoma, the highest and lowest degrees of differentiation (G1 and G4) were absent.
7. Survival of patients with renal cell carcinoma decreased with increasing patient age and tumor necrosis area. It was longer in males compared to females.
8. No statistically significant difference was found in cytoplasmic intensity of AIF in tumor and non-tumor tissue of renal cell carcinoma.
9. Expression of AIF in tumor cell cytoplasm was higher compared to nuclear expression.
10. Nuclear expression of AIF in renal cell carcinoma did not correlate with any of the investigated clinical-morphological indicators: age,

sex, histological variant, degree of carcinoma differentiation, tumor necrosis, TILs, vascular invasion, T, N and M stage, tumor localization and patient survival.

11. Cytoplasmic expression of AIF in tumor cells increased with age and had higher values in the absence of tumor emboli compared to cases with LV invasion.

12. In the cytoplasm of papillary renal cell carcinoma, expression of the apoptosis-inducing factor was higher than that of the clear cell variant and the difference is statistically significant.

13. Expression of AIF in tumor cell cytoplasm at T1 stage was higher compared to T3 stage.

14. RIPK3 in tumor cell cytoplasm decreased compared to adjacent non-tumor tissue.

15. Cytoplasmic level of RIPK3 in tumor cells was higher than nuclear content.

16. Expression of RIPK3 in tumor cell cytoplasm of renal cell carcinoma showed no dependence on the following clinical-morphological indicators: patient sex, histological variant, necrosis area, TILs, T, N and M stage, tumor localization and patient survival.

17. RIPK3 in tumor cell cytoplasm increased with increasing age of patients with renal cell carcinoma and with occurrence of lymphovascular invasion, and decreased with decreasing degree of tumor differentiation.

18. Nuclear expression of RIPK3 in renal cell carcinoma tumor tissue did not correlate with any of the investigated clinical-morphological indicators: patient age and sex, histological variant, degree of differentiation, necrosis area, TILs, vascular invasion, T, N and M stage, tumor localization and patient survival.

VII. CONTRIBUTIONS OF THE DISSERTATION

7.1. Original scientific contributions:

- A complex clinico-morphological and immunohistochemical analysis of renal cell carcinoma was performed in relation to patient survival.
- The processes of apoptosis and necroptosis as prognostic and predictive markers were evaluated immunohistochemically by AIF and RIPK3.

7.2. Scientific contributions of a practical and applied nature:

- The importance of the main clinico-morphological indicators such as age, area of tumor necrosis and vascular invasion as prognostic factors for reduced survival in patients with RCC was evaluated.
- The morphological profile of advanced renal cell carcinoma was confirmed in terms of the degree of differentiation, tumor necrosis, infiltration of TILs, and the risk of death was assessed.
- It was found that there was no correlation between the nuclear expression of the two markers of apoptosis and necroptosis (AIF and RIPK3) and clinico-morphological parameters.
- Cytoplasmic expression of AIF in renal cell carcinoma has been shown to be relevant to lymphovascular invasion and tumor progression.
- The role of RIPK3 cytoplasmic expression in renal cell carcinoma for tumor differentiation and occurrence of lymphovascular invasion was evaluated.

VIII. PUBLICATIONS RELATED TO THE DISSERTATION

Full text articles:

1. **Yanulova N**, Tzaneva M. Role of the apoptosis-inducing factor in physiological conditions and in malignant neoplasms. Varna Medical Forum. 2022;11 (2):78-84.

2. **Yanulova N**. The role of RIPK3 in the process of necroptosis in malignant tumors. Varna Medical Forum. 2023;12(2):55-63.