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Multidimensional approaches in the characterization of renal carcinomas through the integration of omic analyses

SUMMARY OF THE DOCTORAL THESIS

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The doctoral thesis comprises a total of 228 pages, of which 47 represent the General Part. The work includes 85 figures, 19 tables, and 262 bibliographic references.

Note: in the present abstract, the table of contents has been preserved in the same structure as in the full doctoral thesis.

INTRODUCTION

Renal carcinoma is the seventh leading cause of cancer in men and the ninth in women, and it is a major public health problem due to its increasing incidence and biological and clinical heterogeneity [1,2]. The most common histopathological subtypes are clear cell carcinoma (ccRCC), papillary carcinoma (pRCC, type 1 and type 2), and chromophobe carcinoma (chRCC), each with distinct morphological, molecular, and prognostic characteristics [3,4]. In clinical practice, accurate diagnosis and prognostic stratification are often hampered by histological variability and nonspecific clinical presentation, requiring the integration of immunohistochemical and molecular methods in the evaluation of these tumors.

Immunohistochemistry (IHC) remains an indispensable tool for the diagnosis and classification of RCC, complementing conventional histopathological analysis. The classic panel includes epithelial and lineage differentiation markers such as PAX8, CAIX, CD10, CD117, Vimentin, AMACR, RCC, and CK7, which allow differentiation between subtypes and confirmation of renal origin [5,6]. In parallel, markers with prognostic and predictive value, such as VEGF and c-MET, have gained a central role. In ccRCC, VEGF overexpression is directly linked to VHL gene inactivation and activation of hypoxia-induced pathways, correlating with intense tumor angiogenesis and the potential for response to anti-VEGF therapies [7,8]. In pRCC, c-MET overexpression has a dual value: on the one hand, it facilitates differential diagnosis, and on the other hand, it constitutes a validated therapeutic target with clinical applications in MET inhibitors [9,10].

In recent years, microRNAs (miRNAs) have become biomarkers of major interest in RCC, playing both oncogenic and tumor suppressor roles. In this paper, three species of particular relevance were investigated: miR-21-5p, recognized as a classic oncomiR, associated with the proliferation and invasiveness of ccRCC [11]; miR-30c, involved in the regulation of epithelial-mesenchymal transition and with a potential role in sensitivity to targeted therapies [12]; and miR-182-5p, associated with tumor progression and aggressive forms of RCC [13]. The study of the expression of these miRNAs in different biological compartments-tumor tissue, serum, and circulating exosomes- provides a complex perspective on the epigenetic mechanisms involved in carcinogenesis.

Intercompartmental analysis has demonstrated that these microRNAs are not expressed in isolation, but reflect a dynamic equilibrium between the primary tumor and the circulating environment. Correlations between tissue, serum, and exosomal expression may contribute to the definition of non-invasive biomarkers capable of accurately reflecting tumor biology and providing valuable information for early diagnosis, monitoring of progression, and prognosis assessment.

Thus, the integration of immunohistochemistry with microRNA expression analysis and the evaluation of intercompartmental correlations supports a multidimensional approach to the characterization of renal carcinoma, with direct implications for personalized medicine.

CURRENT STATE OF KNOWLEDGE

1. General considerations regarding renal carcinoma

Renal carcinoma is the most frequent malignant tumor of the renal parenchyma and a paradigm of oncologic heterogeneity, from histopathological, molecular, and clinical perspectives. Over the past two decades, advances in the understanding of carcinogenetic mechanisms have profoundly reshaped the approach to this pathology, directing medical practice toward an integrated and multidimensional characterization.

The histopathological classification, according to WHO 2022, comprises three major entities-clear cell carcinoma, papillary carcinoma, and chromophobe carcinoma-supplemented by rare subtypes such as medullary carcinoma, collecting duct carcinoma, and MiT family translocation tumors [4]. This diversity reflects not only distinct morphological features but also specific genetic and molecular foundations, with significant prognostic and therapeutic implications.

At the epidemiological level, the incidence of RCC is steadily increasing, with relevant geographical variations. The discrepancy between the lower incidence and the relatively high mortality reported in Romania, compared to the European average, suggests limitations in early detection and access to innovative therapies, constituting an argument for intensifying efforts in screening and treatment personalization [14].

The etiological factors involved are multiple and complex. In addition to sporadic risk factors—smoking, obesity, hypertension, and occupational exposures [15,16,17]—there are well-defined hereditary contexts such as von Hippel–Lindau, Birt–Hogg–Dubé, HLRCC, and tuberous sclerosis syndromes [18,19,20]. These syndromes confer a particular tumor risk, with early onset, distinct clinicopathological features, and special significance for genetic counseling and family management.

From a clinicopathological perspective, RCC is often asymptomatic in its early stages, with diagnosis frequently being incidental. Classical manifestations are rare, and the clinical picture may be dominated by complex paraneoplastic syndromes. The histopathology of each subtype provides important prognostic information: type 2 papillary carcinoma is associated with aggressive behavior, whereas chromophobe carcinoma generally carries a favorable prognosis.

TNM staging (8th edition AJCC/UICC) and ISUP nuclear grading represent essential benchmarks for prognostic evaluation and therapeutic guidance [4]. Survival varies significantly according to stage, ranging from over 90% in early forms to below 15% in metastatic disease, underscoring the predictive value of these parameters.

Despite progress, the limitations of conventional approaches remain evident: intratumoral heterogeneity, subjectivity of morphological assessment, and the semi-quantitative interpretation of immunohistochemistry. These constraints demand a paradigm shift toward a multidimensional characterization that integrates morphology, immunohistochemistry, molecular data, and digital tools based on artificial intelligence, thereby opening new perspectives for the personalized diagnosis and treatment of renal carcinoma.

2. Molecular Biology of Renal Carcinoma

Renal carcinoma represents a paradigmatic example of molecular heterogeneity in oncology, defining a category of tumors whose clinical evolution and therapeutic response are profoundly influenced by the genomic and epigenomic landscape. Advances achieved over the past two decades have demonstrated that molecular analysis holds not only descriptive value but also direct consequences for differential diagnosis, prognostic stratification, and therapeutic selection.

- **Characteristic Genetic Alterations**

In clear cell carcinoma, inactivation of the VHL gene represents the fundamental initiating event, present in the majority of cases. Loss of VHL protein functionality leads to the accumulation of hypoxia-inducible factors (HIF), with transactivation of pro-angiogenic genes, among which VEGF, PDGF, and GLUT1 play a central role. This molecular mechanism explains the characteristic hypervascular phenotype and the sensitivity of ccRCC to anti-angiogenic therapies. In addition, alterations in genes such as PBRM1, BAP1, and SETD2 define subgroups with distinct prognostic significance, suggesting the existence of alternative pathways of tumor progression [21-27].

Papillary carcinoma exhibits notable differences between its two subtypes. In type 1 pRCC, activating mutations of the c-MET gene induce proliferation and invasion through activation of the hepatocyte growth factor receptor, thereby justifying the development and application of MET

inhibitors. In contrast, type 2 pRCC is frequently associated with FH mutations, defining the context of HLRCC syndrome, known for its clinical aggressiveness and early metastatic potential [10,28,29].

Chromophobe carcinoma, although relatively more indolent, is associated with alterations of the FLCN gene, characteristic of Birt–Hogg–Dubé syndrome, as well as with multiple chromosomal losses. In rare forms, such as medullary carcinoma, loss of SMARCB1/INI1 expression plays a diagnostic role, while MiT family translocation tumors involve fusions at the TFE3 or TFEB level, identified by immunohistochemical profile and confirmed through molecular techniques [30-33].

- **Signaling Pathways Involved**

The mechanisms of renal carcinogenesis converge on intracellular signaling networks that regulate angiogenesis, metabolism, and resistance to cell death. The HIF/VEGF pathway dominates the molecular landscape of ccRCC and underpins the use of anti-VEGF therapies [23]. Dysregulation of mTOR provides proliferative and metabolic advantages, constituting the rationale for the specific inhibitors employed in oncologic practice. In papillary carcinoma, activation of the c-MET/HGF pathway is correlated with an invasive phenotype and unfavorable prognosis. In addition, interactions among the Wnt/TGF-β and PI3K/AKT pathways and epigenetic mechanisms add an additional level of complexity and account for the variability of therapeutic response.

- **The Role of MicroRNAs**

An emerging field in renal molecular biology is represented by microRNAs, small non-coding RNA molecules with post-transcriptional regulatory functions. Altered microRNA expression profiles distinguish between histological subtypes, reflect the degree of tumor aggressiveness, and provide potential as circulating biomarkers. Among them, miR-21-5p behaves as an oncomiR, associated with treatment resistance and unfavorable prognosis; miR-30c-5p has a suppressor role, with reduced expression correlating with tumor progression; and miR-182-5p participates in processes of cellular proliferation and invasion [11,12,13]. Comparative analysis of their expression in tumor tissue, serum, and exosomes enables the identification of inter-compartmental correlations with diagnostic and prognostic value.

- **Clinical and Therapeutic Implications**

The integration of these data has transformed the therapeutic paradigm of renal carcinoma, replacing the exclusively surgical approach with strategies based on targeted therapies (anti-VEGF, MET inhibitors, mTOR inhibitors) and next-generation immunotherapies. Looking forward, multidimensional characterization that combines histopathological analysis, immunohistochemistry, genetic profiling, and microRNA expression, together with artificial intelligence algorithms, holds the promise of providing a framework for personalized diagnosis and treatment tailored to the specific features of each patient.

3. Immunohistochemical Examination in the Evaluation of Renal Carcinoma

Immunohistochemistry (IHC) represents an indispensable tool in the differential diagnosis of renal tumors, complementing classical histopathological examination. The standard panel includes markers such as PAX8, CAIX, CD10, CK7, CD117, AMACR, RCC, and Vimentin, used to differentiate among the main subtypes of renal carcinoma.

In clear cell carcinoma (ccRCC), the strong positive expression of CAIX and CD10, together with PAX8, enables confirmation of renal origin and distinction from other metastatic tumors. In papillary carcinoma (pRCC), CK7 and AMACR are typically positive, whereas chromophobe markers (diffuse CK7, CD117) characterize chromophobe carcinoma (chRCC) [4].

Beyond differential diagnosis, IHC provides valuable prognostic information. VEGF is frequently overexpressed in ccRCC and correlates with increased angiogenesis and biological aggressiveness, forming the basis for anti-angiogenic therapies. In pRCC, c-MET overexpression is relevant both prognostically and as a therapeutic target, being particularly associated with type 1 and with cases showing unfavorable progression.

Therefore, immunohistochemistry, through its dual diagnostic and prognostic role, remains a central pillar in the characterization of renal carcinoma, particularly when integrated with molecular and clinical data.

4. Artificial Intelligence in Pathological Analysis

Artificial intelligence (AI) represents an emerging direction in renal pathology, with major potential to complement traditional histopathological and immunohistochemical evaluation. By integrating machine learning algorithms and digital pathology technologies, AI enables

quantitative analysis of histological and immunohistochemical images, surpassing the limitations of human subjectivity. Current applications include automated segmentation of nuclei and tissue structures, quantification of immunohistochemical marker expression, and identification of subtle patterns of tumor heterogeneity. Platforms such as QuPath and convolutional neural networks (CNNs) have demonstrated utility in the analysis of renal tumors, providing reproducible and objective data. In clear cell carcinoma, AI facilitates the evaluation of angiogenesis and VEGF expression, while in papillary carcinoma it can support the quantification of c-MET expression. Moreover, the integration of imaging analysis with molecular data (including microRNA profiling) outlines predictive models capable of distinguishing tumor subtypes and anticipating clinical evolution [34-37].

The current limitations are related to the standardization of algorithms, the small size of cohorts, and the need for multicenter validation. Nevertheless, through its ability to correlate morphological, immunohistochemical, and molecular data, AI is emerging as an indispensable tool for future multidimensional approaches in the diagnosis and prognosis of renal carcinoma.

PERSONAL CONTRIBUTION

5. Foundations of the Study: Motivation, Aim, Hypothesis, and Objectives

Study Motivation

In the development of renal carcinoma, the genetic component plays an important role, reflected both in hereditary syndromes (VHL, BHD/FLCN, HLRCC/FH, HPRC/MET, TSC, etc.) and in familial predisposition, which significantly increase the risk of renal neoplasia and justify early detection and robust risk stratification in populations with a family history. In parallel, current clinical guidelines recommend partial nephrectomy (to preserve as many functional nephrons as possible) as the preferred option for renal tumors classified as T1 and, selectively, T2-a major benefit that critically depends on early diagnosis, with the potential to avoid total nephrectomy in some patients.

Aim of the Study

The aim of the research was to deepen the understanding of the molecular and phenotypic mechanisms involved in clear cell renal carcinoma, as well as to identify biomarkers with diagnostic, prognostic, and predictive value, integrated into a model applicable to clinical practice.

In this context, the study aims to develop and validate an integrative framework (molecular and immunohistochemical) for the early detection, risk stratification, and monitoring of renal carcinoma, with emphasis on:

- the evaluation of miR-21-5p, miR-30c, and miR-182-5p in tissue, serum, and exosomes (liquid biopsy);
- their correlation with the standard IHC panel (PAX8, CAIX, CD10, CK7, CD117, AMACR ± RCC marker/Vimentin) and with prognostic/predictive markers (VEGF, c-MET);
- their clinical utility for patients with familial risk, with the goal of identifying disease at an early stage, when partial nephrectomy may be favored over radical nephrectomy.

Research Hypotheses

Based on recent data from the scientific literature, the present thesis was built on the following research hypotheses:

1. That the differential expression of microRNAs miR-21-5p, miR-30c, and miR-182-5p is associated with the fundamental mechanisms of renal carcinogenesis (proliferation, invasion, metastasis), supporting their role as diagnostic molecular biomarkers.
2. That the integration of molecular analyses (microRNAs) with the standardized immunohistochemical panel (PAX8, CAIX, CD10, CK7, CD117), supplemented by predictive and prognostic markers (VEGF, c-MET), increases the accuracy of differential diagnosis and enables a more reliable stratification of tumor risk.
3. That circulating microRNAs, detected in exosomes and serum, may serve as clinically valuable "liquid biopsies," useful for diagnosis, postoperative monitoring, and prediction of tumor recurrence.
4. That the correlation of immunohistochemical expression (e.g., VEGF, c-MET) with microRNA expression profiles may outline common functional networks, relevant for understanding the mechanisms of angiogenesis, invasion, and tumor progression.

Main Objectives

1. To analyze the expression profile of miR-21-5p, miR-30c, and miR-182-5p in tumor and non-tumor tissue, serum, and exosomes.

2. To evaluate the diagnostic value of the selected microRNAs, individually and in combination, through ROC performance analysis and clinicopathological correlations.

3. To validate the concept of “liquid biopsy” in renal oncology by comparing microRNA expression in the tumor compartment with that in the circulating compartment (serum and exosomes).
4. To investigate the immunohistochemical expression of the standard panel (PAX8, CAIX, CD10, CK7, CD117, AMACR, RCC, Vimentin) and of the additional markers VEGF and c-MET, highlighting correlations with clinicopathological and molecular parameters.

Secondary Objectives

1. To process and evaluate the histopathological features of surgical specimens/biopsies of renal carcinoma, applying the complete immunohistochemical panel.
2. To isolate and purify microRNAs from tumor and non-tumor tissue, serum, and exosomes, followed by quality and integrity assessment using standardized methods.
3. To perform reverse transcription and quantify the expression of selected microRNAs by RT-qPCR, with subsequent normalization and statistical analysis of the data.
4. To correlate molecular and immunohistochemical results with clinicopathological parameters (age, sex, tumor size, ISUP grade, TNM stage, invasion, metastasis).
5. To test functional associations between VEGF expression and miR-21-5p, and between c-MET expression and miR-182-5p, in order to identify potential biologically relevant interactions.
6. To explore the utility of dynamic monitoring of serum and exosomal microRNA levels in postoperative follow-up and in assessing treatment response.

6. Research Methodology

Case Selection

Cases of patients diagnosed with renal carcinoma (ccRCC, papillary RCC, chromophobe RCC, translocation RCC, and rare forms) between 2019–2024 were selected.

For Study 1:

- retrospective/prospective cohort, selected from the SCAP archive (178 cases);
- inclusion criteria: confirmed diagnoses, available FFPE blocks, absence of neoadjuvant treatment.

For Study 2:

The study was conducted between June 2019 and November 2021 and included 26 paired tissue samples: renal tumor tissue and adjacent non-tumoral tissue, collected at a minimum distance of 5 cm from the tumor lesion to reduce the risk of tumor contamination. The samples were obtained from patients admitted to the Urology Department of the Constanța County Clinical Emergency Hospital "St. Apostle Andrew" (SCJU), who presented with clinical suspicion of renal tumor and underwent radical nephrectomy. In addition, preoperative peripheral blood samples were collected in order to isolate the serum fraction for subsequent analyses, including exosomal microRNA extraction.

The processing of surgical specimens and the establishment of the histopathological diagnosis were carried out within the Clinical Pathology Service (SCAP), in accordance with national good practice protocols in pathology, ensuring the accuracy and reproducibility of the results. The research activity was approved by the Local Ethics Committee for the Approval of Clinical and Research Studies.

All patients included in the study signed informed consent, expressing their agreement for the use of biological samples for scientific purposes and for the processing of personal data in accordance with national and European regulations (GDPR – Regulation (EU) 2016/679). Molecular biology analyses (including total RNA extraction and gene expression quantification by qRT-PCR) and immunohistochemical testing were performed at the Research Center for Morphological and Genetic Studies in Malignant Pathology – CEDMOG, "Ovidius" University of Constanța.

Research Methods

- ***Histopathological Examination of Nephrectomy Specimens***

The histopathological examination of nephrectomy specimens represents the diagnostic standard for renal tumors, involving a rigorous sequence of steps. Following intraoperative collection and transport in compliance with safety regulations, the specimens undergo macroscopic processing and fixation in formalin, followed by the sampling of representative fragments from the tumor, adjacent parenchyma, capsule, perirenal fat, hilum, and, when applicable, the adrenal gland. The fragments are automatically processed, embedded in paraffin, and sectioned at 3–5 μm to allow standard microscopic evaluation with hematoxylin-eosin staining. Histological analysis aims to

identify the tumor type, degree of differentiation, presence of necrosis, invasion of the capsule, perirenal fat, and vascular or adjacent structures, as well as the assessment of resection margins. In parallel, samples dedicated to molecular biology are collected, preserved in RNAlater® solution, and cryogenically stored, thus ensuring material integrity for subsequent gene expression and microRNA analyses.

- ***Immunohistochemical Examination***

Immunohistochemical examination was employed for diagnostic confirmation and molecular characterization of renal tumors. The analysis was based on monoclonal antibodies directed against markers commonly used in renal carcinoma—CK7, PAX8, CD10, and RCC. Formalin-fixed, paraffin-embedded tissue sections were processed through deparaffinization, rehydration, and antigen retrieval, followed by incubation with primary and secondary antibodies, polymer amplification, and chromogenic development using DAB. The slides were counterstained and permanently mounted, resulting in the specific visualization of immunohistochemical marker expression relevant for the subtyping and prognostic assessment of renal carcinoma.

- ***Extraction and Purification of microRNA Species from Serum, Exosomes, and Tissues***

The miRCURY Exosome, miRNeasy Serum/Plasma, and miRNeasy Mini kits provide rapid and efficient methods for the isolation and purification of total RNA, including small RNA fractions (<200 nucleotides), from exosomes, serum, and human tissues.

The **miRCURY** kit operates on the principle of reducing the hydration layer surrounding particles in suspension. By mixing the sample with the precipitation buffer, the hydration layer is diminished, allowing the precipitation of subcellular particles, including those <100 nm, through low-speed centrifugation. In the case of serum samples, residual cells, cellular debris, platelets, and apoptotic bodies are removed. For more efficient elimination of platelets and larger microvesicles, the samples are pre-filtered through 0.2 μm filters.

The **miRNeasy Serum/Plasma** kit is specifically designed for the isolation of total RNA, including RNA species <200 nucleotides, from serum and plasma. The procedure involves phenol/guanidine-based lysis and purification on silica membrane columns. QIAzol, a monophasic reagent containing phenol and guanidinium thiocyanate, ensures complete lysis, denaturation of RNases and proteins, and removal of DNA. The aqueous phase is separated from the organic phase

after chloroform addition and subjected to ethanol precipitation. RNA binds to the silica membrane in the presence of chaotropic salts, is subsequently washed, and eluted with RNase-free water.

The **miRNeasy Mini** kit is intended for the extraction of total RNA from solid tissues and employs a combination of guanidine-phenol lysis and silica column purification. Tissue samples are initially homogenized in QIAzol Lysis reagent, which facilitates complete dissociation of cellular components, inactivation of RNases, and separation of macromolecular fractions. The addition of chloroform and centrifugation of the mixture generates two distinct phases: an upper aqueous phase containing RNA, and a lower organic phase containing DNA and proteins. The RNA from the aqueous phase is precipitated with ethanol and applied to the RNeasy Mini columns. In the presence of chaotropic salts, RNA molecules longer than 18 nucleotides bind to the silica membrane, while contaminants are removed through successive washes. The high-purity RNA is then eluted in RNase-free water, making it suitable for downstream applications such as qRT-PCR, microarray, or next-generation sequencing (NGS).

- ***Assessment of the Quality, Quantity, and Integrity of Extracted RNA***

The assessment of RNA purity and quantity was performed by UV absorbance measurements using a NanoDrop One spectrophotometer. The method is based on the Beer–Lambert law, according to which light absorbance is directly proportional to the concentration of the molecules analyzed.

The wavelengths used for analysis were as follows:

- 260 nm – absorbance of nucleic acids (RNA and DNA)
- 280 nm – absorbance of proteins (particularly tryptophan and tyrosine)
- 230 nm – absorbance of organic contaminants (phenol, guanidines)
- 240 nm and 320 nm – background absorbance, for optical noise correction

Relevant spectral ratios:

- OD260/280 – an indicator of protein contamination; values between 1.8 and 2.2 are considered acceptable.
- OD260/230 – reflects the presence of organic compounds; values above 1.8 indicate high-quality RNA.

In the case of samples with low RNA yield, the assessment of concentration and molecular integrity by UV spectrophotometry becomes limited. For a more accurate analysis of such samples,

the use of the TapeStation 2200 system, quantitative qRT-PCR, or fluorometric quantification is recommended.

Reverse Transcription and Detection of microRNA Species by Real-Time PCR

The TaqMan® Advanced miRNA kit is specifically designed for the quantification of mature microRNA species from a variety of biological samples, including tissues, serum, or plasma. The quantification process involves several successive steps.

In the first step, starting from the isolated total RNA, poly(A) polymerase adds a polyadenylated tail to the 3' end of mature microRNA molecules. Subsequently, in the second step, a specific adaptor is ligated to the 5' end of these molecules. This modification enables efficient initiation of the reverse transcription reaction, performed with the aid of a universal RT primer, resulting in the synthesis of complementary DNA (cDNA) corresponding to the microRNAs in the sample.

In the next step, the obtained cDNA undergoes uniform amplification through a reaction called miR-Amp, which ensures the generation of sufficient product for detection. For specific detection and real-time quantification, Polymerase Chain Reaction (PCR) is performed using TaqMan® MGB probes. In this stage, forward and reverse primers hybridize to complementary sequences of the target cDNA, while the TaqMan® probe-labeled with a reporter dye at the 5' end and a non-fluorescent quencher at the 3' end-binds to the amplified region between the two primers.

When the probe is intact, reporter dye fluorescence is inhibited through the Förster Resonance Energy Transfer (FRET) mechanism. During elongation, DNA polymerase degrades the probe hybridized to the target sequence, thereby separating the reporter dye from the fluorescence quencher. This process results in the release of reporter fluorescence, a signal proportional to the amount of amplicons generated. The observed fluorescence increases progressively over successive amplification cycles and is directly proportional to the initial amount of mature microRNA present in the sample. Since fluorescence detection occurs only if the probe is perfectly complementary to the target sequence and is efficiently amplified, nonspecific reactions are not recorded.

Normalization of Data Obtained by Real-Time Polymerase Chain Reaction

In this study, differentiated normalization strategies were applied to standardize the expression levels of mature microRNA species from renal carcinoma (RCC) tissues and from exosomal serum samples.

In tissue analysis, microRNA expression in RCC tumor samples was compared with that in adjacent normal renal tissue collected from the same patients. For serum samples, normalization of exosomal microRNA levels was performed relative to a control group consisting of healthy individuals, matched for age and sex, with no oncological history or relevant comorbidities.

For the relative quantification of microRNA gene expression, the comparative cycle threshold (Ct) method was applied, using Ct values obtained for the target genes in tumor and serum samples of patients, compared with those in adjacent normal tissues or calibrator sera. The calibrator consisted of a serum sample derived from clinically healthy subjects, matched by age and sex to the patients in the study cohort.

Ct values from each sample were normalized using a stable endogenous reference gene: RNU44 for tissue samples and miR-16-5p for serum samples. Each sample was analyzed in triplicate, both for the microRNAs of interest and for the endogenous controls, in order to ensure reproducibility and accuracy of the results.

Data analysis was performed using the 2^–ΔΔCt method, where ΔΔCt represents the relative difference in target gene expression between the studied samples and the calibrator. The ΔCt value was calculated by subtracting the mean Ct values of the reference gene from the mean Ct values of the target gene.

The results were expressed as relative expression (RQ) or fold change (FC), using the 2^(–ΔΔCt) formula. Thus:

an RQ > 1 indicates overexpression of the target gene;

an RQ < 1 indicates underexpression;

an RQ ≈ 1 suggests comparable expression between the two types of samples.

This relative quantification strategy provides a higher degree of flexibility and reduces experimental variability, allowing for the comparison of microRNA gene expression across different biological conditions and sample types.

Statistical Analysis

The obtained data were analyzed, and graphical representations were generated using the MedCalc statistical software, version 19.0.3. Qualitative variables were expressed as absolute frequency (n) and percentage (%), while quantitative variables were expressed as mean ± standard deviation (SD) or as median and interquartile range (IQR, 25%–75%), depending on data distribution.

The normal distribution of continuous variables was verified using the Kolmogorov–Smirnov test. In cases where the p-value associated with the test was <0.05, the hypothesis of normal distribution was rejected and non-parametric tests were applied.

Comparisons between two groups were performed using the independent samples t-test for data with normal distribution and homogeneous variance, or the Mann–Whitney U test for data with non-normal distribution.

Comparisons among more than two groups were carried out using one-way ANOVA, followed by the LSD post-hoc test for normally distributed data with equal variances, or the Kruskal–Wallis test for non-parametric data.

For categorical variables, the χ^2 (chi-square) test or Fisher's exact test was used, depending on sample size and expected frequencies.

Correlations between two continuous variables were evaluated using Spearman's correlation coefficient (ρ). The threshold for statistical significance was set at $p < 0.05$, below which the null hypothesis was rejected.

The diagnostic feasibility of the investigated microRNAs as potential tissue and serum biomarkers in patients with renal carcinoma (RCC) was assessed using Receiver Operating Characteristic (ROC) curve analysis. Diagnostic performance was characterized by sensitivity (true positive rate) and 1-specificity (false positive rate) at different thresholds. The Youden index (J = sensitivity + specificity – 1) was applied to determine the optimal discrimination threshold and to maximize the area under the ROC curve (AUC).

7. Results and Discussion

Study No. 1

Demographic and Clinicopathological Characteristics of the Studied Cohort

Out of a total of 178 cases, 169 (94.95%) were malignant tumors and 9 cases (5.05%) were benign tumors. Among the malignant neoplasms, clear cell renal carcinoma clearly predominated, being diagnosed in 133 cases (74.7%). Papillary carcinomas accounted for 14 cases (7.9%), while chromophobe carcinoma was identified in 5 cases (2.8%). Rare entities were also identified, including collecting duct carcinoma (1 case, 0.6%), renal neuroendocrine carcinoma (1 case, 0.6%), invasive urothelial carcinomas of low grade (6 cases, 3.4%) and high grade (9 cases, 5.1%), as well as one renal metastasis of adenocarcinoma with digestive tract phenotype (1 case, 0.6%). Regarding benign tumors, the cases included 1 solitary fibrous tumor, 3 oncocytomas, and 5 angiomyolipomas. The analysis of the demographic distribution of the 178 patients included in the study revealed a series of features relevant for understanding the epidemiological context.

With respect to age, 63 patients (35.4%) were ≤65 years old, while the remaining 115 patients (64.6%) were older than this threshold. This distribution confirms the trend reported in the international literature, according to which the incidence of renal cancer increases progressively with age, with a peak diagnosis in the sixth and seventh decades of life.

From a demographic perspective, the majority of patients—110 cases (61.8%)—originated from urban areas, while 68 cases (38.2%) came from rural areas. This difference may reflect easier access of the urban population to medical services and imaging diagnostic methods, favoring the detection of renal tumors at earlier stages.

Regarding patient sex, the studied cohort included 71 women (39.9%) and 107 men (60.1%), maintaining the male predominance ratio described in most epidemiological studies, where men are affected approximately twice as often as women.

An aspect of epidemiological and clinical interest in renal tumor pathology is the distribution of cases according to localization in the left or right kidney. Analysis of the patient cohort included in the study revealed a relatively balanced distribution between the two sides, without statistically significant differences, which is consistent with data reported in the literature. This relative symmetry suggests that the carcinogenetic mechanisms involved do not exhibit lateralized tropism but are determined by systemic risk factors (hypertension, obesity, smoking, genetic predispositions), complemented by local microvascular and anatomical particularities.

Tumor size not only reflects disease extent but also represents an independent prognostic factor, with patients presenting lesions <4 cm showing significantly higher survival rates compared to those with larger tumors. Moreover, conservative surgery is more often feasible in small tumors, increasing the likelihood of preserving renal function. In the studied cohort, the maximum tumor size (cm) was distributed as follows: ≤4 cm in 50 cases (30.67%); ≤7 cm in 68 cases (41.72%); ≤10 cm in 32 cases (19.63%); and >10 cm in 13 cases (7.98%).

Histopathological and Immunohistochemical Analysis of Renal Tumors

Renal tumor pathology is characterized by significant histogenetic and morphological diversity, reflected both in macroscopic features and in microscopic and immunohistochemical profiles. In the comprehensive evaluation of these neoplasms, the macroscopic analysis of the surgical specimen plays a central role, enabling the correlation of tumor dimensions, cut surface appearance, and relationship with perirenal structures with the histological and molecular profile.

Immunohistochemistry (IHC) is indispensable in the differential diagnosis of renal tumors, particularly in cases with atypical or overlapping morphological features. IHC marker panels facilitate not only positive and differential diagnosis but also the identification of rare variants, with direct impact on prognosis and treatment.

Within the cohort of 178 patients examined between 2019–2024, an extended immunohistochemical analysis was performed for 68 cases, to which two additional cases from 2025 were included, representing:

- mucinous tubular and spindle cell carcinoma (MTSCC);
- acquired cystic disease-associated renal cell carcinoma (ACKD-RCC).

Thus, the immunohistochemical analysis was performed on a total of 70 cases, selected based on morphological particularities and the need for differential diagnosis.

The studied cohort consisted predominantly of clear cell renal carcinomas and papillary carcinomas, confirming the predominance of these two histological subtypes in renal tumor pathology. The application of the classical immunohistochemical panel (PAX8, CK7, CD10, CD117, RCC, Vimentin, AMACR) enabled validation of the differential diagnosis and definition of the immunophenotypic architecture of each case, while also providing prognostic stratification information. Complementing this, the analysis of VEGF and c-MET expression brought additional

clinically relevant data, highlighting the role of molecular biomarkers in the integrated characterization of RCC.

In clear cell carcinoma, VEGF overexpression correlated with an increased degree of tumor angiogenesis, reflecting the activation of the HIF–VEGF pathway characteristic of this entity. This finding confirms the importance of VEGF as both a prognostic marker and a major therapeutic target, given the demonstrated sensitivity of ccRCC to anti-angiogenic therapies. In contrast, in papillary carcinoma, the dominant marker proved to be c-MET, whose elevated expression levels were associated with a proliferative and invasive phenotype, particularly in the papillary type 1 subtype. This profile suggests the involvement of MET/HGF pathway activation in the biological progression of pRCC and justifies the development of anti-MET targeted therapies for cases with aggressive or refractory evolution.

Therefore, the correlation of classical immunohistochemistry with the evaluation of VEGF and c-MET expression strengthens the value of the morpho-molecular characterization of renal carcinoma, providing a more precise framework for diagnostic and prognostic interpretation. This integration allows not only the delineation of histological subtypes but also guidance toward personalized therapeutic strategies, in accordance with the biological particularities of each tumor entity.

Although PAX8 is considered a sensitive and specific nuclear marker for epithelial renal tumors, it must be emphasized that there are particular situations of immunohistochemical negativity, especially in renal metastases from non-renal neoplasms. In such cases, the absence of PAX8 expression may guide the diagnosis toward an extrarenal origin, in contrast to primary renal tumors where the marker is generally positive. Rare exceptions of PAX8-negative primary renal carcinoma have also been reported, particularly in poorly differentiated or dedifferentiated forms, which justifies the use of an extended immunohistochemical panel for diagnostic confirmation (in correlation with CAIX expression). The integration of these aspects into current practice is of major importance for differentiating between primary and metastatic renal tumors, preventing misclassification and ensuring appropriate therapeutic management.

Study No. 2

Analysis of microRNA Gene Expression

The analysis of microRNA gene expression was performed using the comparative ΔΔCt method. For each sample, Ct values were normalized relative to the selected reference genes, obtaining ΔCt values. The mean ΔCt values were then used to compare statistical differences between the sample groups (NRT vs. RCC), providing a robust estimate of expression variation between the analyzed conditions.

The relative expression of each microRNA was calculated as an expression ratio using the 2^(–ΔΔCt) formula, which allows interpretation of expression changes in terms of fold change. To provide a more robust characterization of data distribution, the median RQ values were used to assess the degree of overexpression or underexpression of each microRNA, indicating how many times the expression level was increased or decreased in renal carcinoma patients compared to the control group.

- **Gene hsa-miR-21-5p**

In the present study, the expression of the hsa-miR-21 gene showed a statistically significant increase in renal tumor tissue compared with adjacent normal samples, with a mean ΔCt of 5.25 versus 1.79 ($P < 0.001$). This difference indicates a tumor overexpression profile of miR-21, suggesting its involvement in mechanisms of tumor progression and invasion. At the circulating level, a significant increase in miR-21 expression was observed both in the serum of RCC patients (5.92; 95% CI: 4.67–7.69) and in serum-derived exosomes (5.29; 95% CI: 4.24–6.67), compared with the control group (1.61; 95% CI: 1.06–3.58 and 1.92; 95% CI: 1.18–2.85, respectively; $P < 0.001$). The concomitant presence of this microRNA in serum and in the exosomal fraction highlights the role of exosomes as key carriers for transport and stabilization of circulating microRNAs, facilitating intercellular communication and promoting pro-neoplastic processes. Relative expression of the miR-21 gene revealed an overexpression of approximately 2.79-fold in tumor tissue (2.79; 95% CI: 1.76–3.65), 4.90-fold in serum (4.90; 95% CI: 3.01–6.48), and 3.44-fold in serum exosomes (3.44; 95% CI: 2.48–4.40), confirming its potential as both a tissue and circulating biomarker in renal carcinoma.

- **Gene hsa-miR-30c**

The expression of the miR-30c gene was significantly reduced in renal tumor tissue compared with adjacent normal tissue, with mean ΔCt values of 3.69 (95% CI: 3.07–4.61) versus 6.17 (95% CI: 5.76–7.15; $P < 0.001$). Regarding the circulating component, miR-30c showed a significant decrease both in serum and in serum-derived exosomes of RCC patients compared with the control group. The serum level was 3.76 (95% CI: 2.86–4.61) compared with 6.23 in the control group (95% CI: 5.06–7.48; $P = 0.002$), while the exosomal level was 3.24 (95% CI: 2.89–3.71) compared with 5.53 in the control group (95% CI: 4.37–6.61; $P = 0.001$). Relative expression analysis demonstrated an underexpression of approximately 2.55-fold in tumor tissue (95% CI: 2.01–3.09), 1.54-fold in serum (95% CI: 1.39–2.23), and 1.37-fold in serum exosomes (95% CI: 1.09–2.23), confirming the potential of miR-30c as both a tissue and circulating biomarker in renal carcinoma.

- **Gene hsa-miR-182-5p**

Regarding the expression of the hsa-miR-182-5p gene, a statistically significant increase was observed in renal tumor tissue compared with adjacent normal samples, with a mean ΔCt of 4.26 (95% CI: 1.09–2.01) versus 1.79 (95% CI: 0.51–0.85; $P < 0.0001$). This difference suggests a tumor overexpression profile of miR-182-5p. At the circulating level, the increase in expression was less pronounced than in tissue: 1.30 (95% CI: 1.14–1.71) in serum compared with the control group at 0.91 (95% CI: 0.55–1.06; $P = 0.0012$). In exosomes, expression showed a more evident increase, with values of 2.36 (95% CI: 1.67–3.23) in RCC patients compared with 1.51 (95% CI: 1.21–1.97; $P = 0.005$) in the control group. Relative expression analysis indicated an overexpression of miR-182-5p estimated at 2.55-fold in tumor tissue (95% CI: 2.01–3.09), 1.54-fold in serum (95% CI: 1.39–2.23), and 1.37-fold in serum exosomes (95% CI: 1.09–2.23), confirming the potential of miR-182-5p as both a tissue and circulating biomarker in renal carcinoma.

Diagnostic and Prognostic Feasibility of microRNA Species

Receiver operating characteristic (ROC) analysis was used to evaluate the diagnostic and prognostic feasibility of the selected microRNA species as tissue and circulating biomarkers in renal carcinoma.

ROC curves allow the visualization of sensitivity (true positive rate) and specificity (false positive rate) across different threshold values for each analyzed microRNA. The diagnostic performance of each microRNA, namely the ability to discriminate between tumor and non-tumor tissue, was expressed by the area under the ROC curve (AUC).

Given that microRNA expression results are expressed as continuous values (ΔCt), an optimal cut-off value was established for each biomarker. This was determined by identifying the point on the ROC curve where the Youden index (sensitivity + specificity – 1) reached its maximum value, indicating the best balance between sensitivity and specificity.

The results indicate that the expression levels of the selected microRNAs may serve as useful tools in differentiating tumor tissues from adjacent normal tissues, as well as in discriminating serum and exosomal samples from controls in patients with renal cell carcinoma (RCC). The diagnostic performance of these biomarkers demonstrated sensitivity values ranging from 57.69% to 92.31%, while specificity varied between 65.38% and 96.15%.

Moreover, the diagnostic performance of biomarker combinations showed sensitivity values ranging from 80.77% to 96.15%, while specificity varied between 88.46% and 100%.

It was observed that miR-21-5p showed an area under the ROC curve (AUC) of 0.87 in tissue analysis, associated with a sensitivity of 65.38% and a specificity of 96.15% ($P < 0.001$). In the circulating compartment, the values were comparable: in exosomes, sensitivity was 92.31% and specificity 73.08%, with an AUC of 0.87, while in serum, sensitivity reached 92.31% and specificity 65.38%, corresponding to an AUC of 0.83 ($P < 0.001$).

miR-30c showed an AUC of 0.81 in tissue analysis, associated with both sensitivity and specificity of 80.77% ($P < 0.001$). In the circulating compartment, the performances were comparable: in exosomes, the AUC was 0.76, with a sensitivity of 76.92% and a specificity of 73.08%, while in serum, the AUC was 0.74, corresponding to a sensitivity of 76.92% and a specificity of 69.23% ($P < 0.001$).

At the tissue level, miR-182 demonstrated an AUC of 0.86, associated with a sensitivity of 92.31% and a specificity of 69.23% ($P < 0.001$). In the circulating compartment, performances were comparable: in exosomes, the AUC was 0.72, with a sensitivity of 57.69% and a specificity of 84.62%, while in serum, the AUC reached 0.76, corresponding to a sensitivity of 73.08% and a specificity of 76.92% ($P < 0.001$).

To assess the diagnostic feasibility of microRNA combinations in tissue, exosomes, and serum, predictive models were generated using logistic regression. The combinations of the three studied microRNAs (miR-21, miR-30c, and miR-182) demonstrated superior performance compared to each individual analysis.

At the tissue level, the AUC value was 0.94, with a sensitivity of 96.15% and a specificity of 92.31% ($P < 0.001$). In the circulating compartment, for the combination of microRNAs in exosomes, the AUC was 0.93, associated with a sensitivity of 80.77% and a specificity of 100% ($P < 0.001$), while for the combination of microRNAs in serum, the AUC was 0.95, with a sensitivity of 92.31% and a specificity of 88.46% ($P < 0.001$).

Correlation of microRNA gene expression with clinicopathological variables

The clinical relevance of the differential expression of the microRNA species analyzed in this study (miR-21-5p, miR-30c, and miR-182-5p) was evaluated in relation to a series of clinicopathological parameters, including sex, age, primary tumor localization, tumor differentiation grade (ISUP), venous invasion, capsular invasion, perineural invasion, lymph node status (pN), presence of metastases (pM), and tumor size.

Statistical analysis did not reveal any significant correlation between the relative expression of the studied microRNAs (across all three biological compartments: tissue, exosomes, and serum) and the following parameters: sex, age, intrarenal topographic localization of the tumor (upper, middle, or lower pole), maximum tumor size, or perineural invasion. These observations suggest that the expression of these microRNAs is relatively independent of demographic characteristics and intrarenal distribution but may be influenced by the molecular tumor phenotype and biological aggressiveness.

However, statistically significant correlations were identified, with potential relevance for prognostic stratification and for understanding tumor mechanisms:

- Exosomal miR-21-5p showed a significant association with renal tumor laterality (right vs. left kidney), with a p-value of 0.02. This finding may suggest an exosomal secretion pattern influenced by the local microenvironment or by anatomical and vascular differences between the two kidneys, a hypothesis that nevertheless requires further validation.

- Tissue-expressed miR-182-5p was also significantly associated with tumor laterality ($p = 0.02$), as was exosomal miR-182-5p, with a p-value of 0.03. The shared correlation across these biological compartments underscores the consistency of miR-182-5p expression in relation to tumor localization and highlights the potential of the exosomal fraction as a non-invasive biomarker.
- Another significant correlation was observed between tissue miR-182-5p expression and lymph node involvement (pN stage), with a p-value of 0.04. This finding suggests a potential role of miR-182-5p in promoting lymphatic dissemination in RCC, supporting the literature that implicates it in migration and invasion processes in other cancer types.

Correlations between the Expression Compartments of miRNAs and Their Biological Implications in RCC

The differential expression of miRNAs across multiple biological compartments (tumor tissue, exosomal, and plasma) provides valuable opportunities for the identification of molecular biomarkers with clinical applicability, both for early diagnosis and for monitoring the progression of neoplasia. In this study, Spearman's correlation coefficient analysis was applied to assess the consistency of miR-21, miR-30c, and miR-182 expression between the tissue compartment and the circulating compartment (serum and exosomes).

The results demonstrated statistically significant correlations between miRNA expression in tumor tissue and that in exosomes or serum, with the strongest associations observed between tissue and exosomal compartments:

- miR-21 tissue – miR-21 exosomes: $\rho = 0.662$; $p = 0.0002$
- miR-30c tissue – miR-30c exosomes: $\rho = 0.578$; $p = 0.0020$
- miR-182 tissue – miR-182 exosomes: $\rho = 0.656$; $p = 0.0003$

These strong correlations support the hypothesis that exosomes act as faithful biological carriers reflecting the molecular profile of the tumor tissue. Since exosomes are actively released by tumor cells and contain specific molecules (RNA, proteins, lipids) involved in intercellular communication, the consistency between tissue and exosomal expression highlights their potential to serve as non-invasive biomarkers with prognostic or predictive value in renal cell carcinoma.

With regard to the serum compartment, significant correlations were also noted, particularly for:

- miR-30c tissue – miR-30c serum: $\rho = 0.688$; $p = 0.0001$
- miR-182 tissue – miR-182 serum: $\rho = 0.623$; $p = 0.0007$

These data reinforce the validity of circulating miRNA profiles as a faithful reflection of tumor expression, while also representing an easily accessible source for molecular evaluation in RCC patients. However, the correlation between miR-21 tissue – serum ($\rho = 0.326$; $p = 0.1042$) did not reach the threshold of statistical significance, which may suggest a differential release of miR-21 into the serum, possibly through alternative extracellular mechanisms (e.g., protein aggregates, non-exosomal vesicles) or due to its faster degradation in circulation.

Overall, these correlations support the hypothesis of a functional relationship between tumor expression and the circulating miRNA signature, strengthening the argument for the use of exosomes and serum as complementary sources of biomarkers in RCC. The present observations are consistent with recent studies that have demonstrated the high stability of miRNAs within exosomes, as well as their potential in the early detection and prognostication of various cancer types.

8. Discussions and Final Conclusions

The integrated analysis of the data obtained in this thesis, by correlating microRNA expression with immunohistochemical results, has outlined new perspectives on the molecular and phenotypic mechanisms involved in clear cell renal carcinoma. The investigation of the selected microRNAs (miR-21-5p, miR-30c, and miR-182-5p) demonstrated their essential role in proliferation, invasion, and metastasis, as well as their potential as circulating biomarkers capable of providing diagnostic and prognostic information. In parallel, the application of the standardized immunohistochemical panel (PAX8, CAIX, CD10, CK7, CD117), supplemented with predictive and prognostic markers (VEGF, c-MET), reinforced the translational value of the study, contributing to improved accuracy in differential diagnosis and in risk stratification of renal tumors.

The results show that the integration of molecular and immunohistochemical analyses not only enhances the understanding of the etiopathogenesis of renal carcinoma but also provides

clinically valuable tools with direct applicability in personalized medicine. Since PAX8, a well-established marker for renal tumors, may lose its expression particularly in metastases, the necessity of correlating it with other immunohistochemical markers and microRNA expression data becomes evident in order to ensure an accurate and comprehensive diagnosis. Thus, the findings outline an integrative model in which molecular and immunohistochemical biomarkers act complementarily, paving the way for multimarker algorithms that can support therapeutic decisions and patient monitoring.

1. The differential expression profile of miR-21-5p, miR-182-5p, and miR-30c supports their role as molecular biomarkers in renal carcinoma.

2. miR-21-5p and miR-182-5p are consistently overexpressed across all compartments (tissue, serum, and exosomes), suggesting an oncogenic role in tumor development and progression.

3. miR-30c is underexpressed, indicating a potential tumor suppressor function that is lost in RCC.

4. Exosomes represent a stable and informative source of molecular biomarkers, showing significant correlations with the tumor compartment.

5. The combination of the three microRNAs provides excellent diagnostic performance, with AUC values exceeding 0.90, reinforcing the concept of an integrated molecular testing panel.

6. The associations identified with clinicopathological variables highlight the potential of these molecules in prognostic stratification and the personalization of therapeutic management.

7. Monitoring the dynamics of these miRNAs in serum or exosomes may provide valuable insights into postoperative tumor recurrence or treatment response, suggesting their applicability in the longitudinal clinical follow-up of patients with renal carcinoma.

8. Immunohistochemical tests remain an essential component in the differential diagnosis of renal tumors, particularly in their subclassification. The classical antibody panel used in routine anatomical pathology includes PAX8, CAIX, CD10, CK7, CD117, AMACR, and RCC marker, each with specific utility in distinguishing between histological subtypes (e.g., ccRCC vs. papillary

vs. chromophobe). In particular, strong and diffuse expression of CAIX is highly suggestive of ccRCC, whereas CK7 positivity is typical for papillary renal carcinoma.

9. In addition to these standard markers, the immunohistochemical analysis of VEGF (Vascular Endothelial Growth Factor) and c-MET (the tyrosine kinase receptor encoded by the MET proto-oncogene) provides valuable insights into tumor angiogenesis and invasive potential. Recent studies, including observations from the analyzed cohort, have shown correlations between VEGF expression and ISUP nuclear grade, as well as between c-MET overexpression and advanced disease stages, suggesting their role as predictive factors of aggressive tumor behavior.

10. By correlating the expression of immunohistochemical markers with the levels of the analyzed microRNAs, a potential functional parallel emerges between VEGF overexpression and miR-21-5p, both being associated with angiogenesis and tumor progression. Similarly, miR-182-5p, which is involved in stress response regulation and DNA repair, may have functional links with c-MET activation, a pathway implicated in cell motility and metastasis. These observations require further validation but open promising directions for integrative multi-omic research.

Therefore, the results of this study contribute to strengthening the concept of "liquid biopsy" in renal oncology and support the integration of microRNAs and immunohistochemical markers (including VEGF and c-MET) into diagnostic and prognostic algorithms. The integrated molecular–morphological approach can guide precision medicine in renal carcinoma, ensuring improved patient stratification and more effective disease monitoring.

9. Originality and Innovative Contributions of the Thesis

Originality of the Thesis

- Innovative integrative approach – the thesis proposes the correlation of microRNA expression in multiple biological compartments (tumor tissue, serum, and plasma-derived exosomes) with clinicopathological and immunohistochemical data, within a unified model applicable in clinical practice.
- First analysis on a Romanian cohort – this is the first time that, within a local population, miR-21-5p, miR-30c, and miR-182-5p are simultaneously investigated alongside VEGF

and c-MET markers, integrated into a complex predictive model for diagnosis, prognosis, and monitoring.

- Methodological innovation through liquid biopsy – the use of modern technologies for exosome extraction and molecular profiling represents a frontier element in the non-invasive diagnosis of renal cancer and provides additional scientific value to the thesis.
- Exploration of the tumor microenvironment – the study offers an original perspective by evaluating microRNAs extracted not only from tumor tissue but also from adjacent non-tumoral areas, an essential aspect for understanding the molecular persistence of the disease and the risk of early recurrence.
- Feasibility of dynamic monitoring – the thesis demonstrates the possibility of evaluating microRNAs in blood samples collected pre- and postoperatively, providing a non-invasive, repeatable, and accessible source for the longitudinal follow-up of patients. The circulating analysis of these biomarkers reflects the real-time molecular evolution of the tumor, opening promising perspectives for the implementation of renal liquid biopsy in clinical practice.

Study Limitations

A major limitation of the study is the relatively small sample size, which may affect the statistical robustness of certain correlations. In addition, the study focused primarily on a dominant histological subtype (ccRCC), without extending the analysis to other rare or mixed entities of renal carcinoma. The absence of external validation (independent cohorts or multicenter studies) restricts the generalizability of the findings. Furthermore, the technologies employed (qPCR, conventional IHC), although standardized, do not fully capture the functional complexity of the molecular networks involved in tumorigenesis.

Future Research Directions

Considering the results obtained in this thesis, future research perspectives in the field of renal pathology are shaped along several complementary directions, aimed at expanding both the applicability of microRNAs and their clinical value in the diagnosis, prognosis, and monitoring of patients with renal diseases.

- A first direction concerns the study of the dynamics of circulating microRNAs after nephrectomy. The longitudinal analysis of serum levels of miR-21-5p, miR-30c, and miR-182-5p before and after surgical excision could provide essential data regarding the value of these biomarkers in postoperative monitoring, the early identification of recurrences or metastases, as well as in stratifying patients according to evolutionary risk [261]. This approach would significantly enhance clinical practice through its minimally invasive nature and its potential to complement the information provided by follow-up imaging.
- A second direction is the extraction and analysis of microRNAs from urinary exosomes, collected both preoperatively and postoperatively. Urine represents an accessible and non-invasive biological fluid, while exosomes contain a stable repertoire of microRNAs that reflect intratumoral molecular processes [262]. Investigating changes in the expression of miR-21-5p, miR-30c, and miR-182-5p in urinary exosomes before and after surgical intervention could open new perspectives in minimally invasive monitoring of patients, with the potential to become complementary tests in routine clinical practice.
- It also appears relevant to extend investigations to benign renal tumors, such as oncocytoma or angiomyolipoma, in order to evaluate whether the same microRNAs (miR-21-5p, miR-30c, miR-182-5p) exhibit expression changes in these entities. Comparing the microRNA profiles between benign and malignant lesions could contribute to the development of differential algorithms, extremely useful in clinical situations where diagnosis is difficult based solely on conventional imaging or histopathological criteria.

In addition, validating these results in larger, multicenter cohorts, as well as integrating the obtained data with global transcriptomic, proteomic, and immunohistochemical information, would enable the construction of complex predictive models. The use of artificial intelligence and machine learning algorithms could facilitate the identification of robust multimarker signatures with direct clinical applicability.

Overall, the future research directions stem from the central hypothesis confirmed by this thesis: microRNAs not only reflect the fundamental processes of renal carcinogenesis but can also be exploited as versatile biomarkers, detectable in biological fluids and complementary to

immunohistochemical markers, thereby opening real prospects for the implementation of personalized medicine in renal tumor pathology.

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List of Publications

Full Articles Published in ISI Journals with Impact Factor:

1. **Burlacu Ionuț**; Așchie, M.; Cozaru, G.C.; Deacu, M.; Vizireanu, G.M.; Mitroi, A.N.; Mitroi, A.F.; Brînzan, C.S. The miR-21-5p, miR-30c-5p, and miR-182-5p as Biomarkers in Clear Cell Renal Cell Carcinoma: A Southeastern Romanian Cohort Study. *Genes* 2025, *16*, 650. https://doi.org/10.3390/genes16060650, indexat Web of Science (SCIE), PubMed, Scopus, factor de impact 2,4.

2. **Burlacu Ionuț** , Mariana Aşchie, Mădălina Boşoteanu, Gabriela Izabela Bălţăţescu, Alexandra Dinu Mucinous Tubular and Spindle Cell Carcinoma of the Kidney: A Rare Renal Neoplasm—Case Report and Literature Review revista Reports MDPI, indexat Web of Science (ESCI), PubMed, factor de impact 0,6.

Scientific Articles Published in Full in BDI-Indexed Journals – First Author/Corresponding Author/Last Author/Authors with Equal Contribution:

1. **Burlacu Ionuț** , Așchie Mariana, Deacu Mariana, Cozaru Georgeta Camelia, Brînzan Costel Stelian, Cojocaru Oana, Dinu Alexandra, Talpeș Raluca, Iorga Ionuț-Ciprian, Enciu Manuela Acquired cystic disease associated renal cell carcinoma clinically manifested as a retroperitoneal hematoma – brief presentation and review of the literature, ARS Medica Tomitana 2025, 2(31): pag.59-64, doi: 10.2478/arsm-2025-0009.

2. Gabriela Izabela Bălțățescu, Nicolae Dobrin, **Burlacu Ionuț.** The Role and Financial Implications of Digital Pathology for the System Health. Ovidius University Annals. Economic Sciences Series 2020;20(1):845-851, jurnal indexat BDI (EBSCO, DOAJ, RePEc, ERIH PLUS, Cabell's Directories).