UNIVERSITY OF MEDICINE AND PHARMACY
„CAROL DAVILA", BUCHAREST
DOCTORAL SCHOOL
MEDICINE DOMAIN

THE IMPACT OF DEVELOPING NEW METHODS FOR ASSESSING SURVIVAL IN PATIENTS WITH COLORECTAL CANCER

ABSTRACT OF THE DOCTORAL THESIS

PhD Supervisor:

Prof. Univ. Dr. GINGHINĂ OCTAV

PhD Student:

Dr. ZAMFIR MARIUS - COSMIN

2026

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List of abbreviations

APC – *Antigen-presenting cells* (celule prezentatoare de antigen)

BRAF –Proto-oncogen B-Raf, kinază serin/treonin

BSA – *Bovine Serum Albumin* (albumină serică bovină)

CAF(s) – *Cancer-associated fibroblasts* (fibroblaste asociate cancerului)

CD – Cluster de diferențiere

cfDNA/ctDNA – *Cell-free DNA* (ADN liber circulant)

cfNA – *Cell-free nucleic acids* (acizi nucleici liberi circulanți)

CH – Hematopoieză clonală

CI – *Confidence interval* (interval de încredere)

CIMP – *CpG Island Methylator Phenotype* (Fenotip metilator al insulelor CpG)

CRC – Cancer colorectal

CRT – Chemoradioterapie

CSCs – *Cancer stem cells* (celule stem tumorale)

CT – Centrul tumoral

CTLA-4 – *Cytotoxic T-lymphocyte-associated protein 4* (Proteină asociată limfocitelor T citotoxice 4)

CTCs – Celule tumorale circulante

ECM – *Extracellular matrix* (Matrice extracelulară)

EGFR – *Epidermal growth factor receptor* (Factorul de creștere epidermal)

FFPE – *Formalin-fixed paraffin-embedded* (Țesut fixat în formol și inclus în parafină)

FOLFOX – 5-Fluorouracil, Leucovorin, Oxaliplatin

H&E – Hematoxylină & eozină

IHC – Imunohistochimie

ICIs – *Immune checkpoint inhibitors* (Inhibitori ai punctelor de control imun)

IL – Interleukină

IM – *Invasive margin* (margine invazivă)

KRAS – *Kirsten rat sarcoma viral oncogene* (Oncogenul viral sarcom Rat Kirsten)

LNR – *Lymph node ratio* (Raportul ganglionilor limfatici)

MACS – *Magnetic Activated Cell Sorting* (Sortare celulară activată magnetic)

MAP2K1 – Mitogen-activated protein kinase kinase 1

MINEN – *Mixed neuroendocrine neoplasm* (Neoplasm mixt neuroendocrin)

MMR – *Mismatch repair* (Reparare a erorilor de împerechere)

MRD – *Minimal residual disease* (Boala minimă reziduală)

MSI – *Microsatellite instability* (Instabilitate microsatelitară)

MSI-H – *Microsatellite instability-high* (Instabilitate microsatelitară înaltă)

NGS – *Next-generation sequencing* (Secvențiere de nouă generație)

NK cells – Celule *Natural killer*

NRAS – *Neuroblastoma RAS viral oncogene* (Oncogenul viral sarcom neuroblastom)

PBS – *Phosphate-buffered saline* (Tampon fosfat salin)

PCR – *Polymerase chain reaction* (Reacție de polimerizare în lanț)

PD-1 – *Programmed cell death protein 1* (Proteină 1 de moarte celulară programată)

PD-L1/2 – *Programmed death-ligand 1/2* (Ligandul 1/2 al proteinei pentru moarte celulară programată)

PI3K – Fosfatidilinozitol-3-kinază

PIK3CA – Subunitatea catalitică alfa a fosfatidilinozitol-3-kinazei

ROS1 – Proto-oncogena ROS1

SSC – *Side scatter* (lumina dispersată)

SNVs – *Single-nucleotide variants* (Variante mononucleotidice)

STAT – *Signal transducer and activator of transcription* (Transductor de semnal și activator al transcripției)

TAMs – *Tumor-associated macrophages* (Macrofage asociate tumorilor)

TANs – *Tumor-associated neutrophils* (Neutrofile asociate tumorilor)

TGF-β – *Transforming growth factor beta* (Factor de creștere transformant beta)

TILs – *Tumor-infiltrating lymphocytes* (Limfocite infiltrante tumorale)

TME – *Tumor microenvironment* (Micromediu tumoral)

TNM – Sistem internațional de stadializare: *Tumor* (tumoră), *Node* (nodul limfatic), *Metastasis* (metastază)

TP53 – gena care codifică pentru proteina p53 (Gardianul genomului)

VEGF – *Vascular endothelial growth factor* (Factorul de creștere endotelial vascular)

Introduction

Colorectal cancer currently represents one of the leading causes of morbidity and mortality worldwide, consistently ranking among the most frequent malignancies in terms of both incidence and cancer-related death. Over recent decades, rapid advances in tumor biology, diagnostic techniques, and therapeutic strategies have profoundly changed the way this disease is understood and managed. Nevertheless, despite remarkable progress, patient survival remains heterogeneous, being influenced by a multitude of biological, clinical, and therapeutic factors that are insufficiently integrated into current prognostic assessment models. (1)

The motivation for choosing this research topic derives from the need for a more nuanced understanding of the factors that influence survival in colorectal cancer, beyond classical staging parameters. In current clinical practice, discrepancies are frequently observed between disease stage and the actual clinical course of patients, suggesting the existence of subtle biological mechanisms that remain insufficiently explored. This observation led to the interest in developing and integrating modern evaluation methods—such as lymph node yield analysis, liquid biopsy, and characterization of the tumor microenvironment—capable of providing a more accurate image of tumor behavior. (2)

The importance and topicality of this subject are supported by the transition of oncology toward a personalized medicine model, in which therapeutic decisions are guided not only by anatomopathological characteristics, but also by the molecular and immunological profile of the tumor. The novelty of the thesis lies in the integrative approach to apparently distinct parameters—surgical, molecular, and immunological—and in the evaluation of their cumulative impact on survival. This multidimensional perspective reflects current trends in international research, where the focus is shifting from a static assessment of disease toward a dynamic understanding of the tumor–host interaction. (3-5)

The topic is directly aligned with the concerns of the international scientific community, where numerous studies investigate the role of liquid biopsy in disease monitoring, the significance of lymph node parameters in staging, and the importance of the tumor microenvironment in determining therapeutic response. At national and institutional level, this research direction is supported by the activity of the team within which modern protocols for molecular

and immunological analysis have been developed, as well as clinical studies focused on optimizing the management of patients with colorectal cancer.

The research hypothesis of this thesis is based on the premise that the integration of multiple biomarkers—including lymph node yield parameters, data obtained through liquid biopsy, and characteristics of the tumor microenvironment—may lead to a prognostic assessment superior to conventional models, allowing a more precise stratification of patients and a more appropriate adaptation of treatment. (5-9)

The aim of the present research is to evaluate the impact of modern methods of tumor characterization on the assessment of prognosis and survival in patients with colorectal cancer, with the purpose of optimizing therapeutic stratification and individualizing oncological management.

General objectives:

• To develop tools through which radical surgical intervention can be objectified and confirmed, by identifying objective elements capable of assessing prognosis and modulating treatment.

• To identify biological factors with predictive and prognostic potential that may contribute to the optimization of personalized therapeutic strategies.

• To analyze circulating biomarkers identified through liquid biopsy and to evaluate their potential use for monitoring disease evolution and treatment response in patients with colorectal cancer.

• To characterize the components of the tumor microenvironment and to evaluate their influence on tumor progression and oncological prognosis.

Specific objectives:

• To evaluate the influence of relevant clinicopathological parameters, such as lymph node status, tumor molecular profile, or histological characteristics, on patient prognosis.

• To analyze the correlations between biomarkers detected through liquid biopsy and disease stage, therapeutic response, recurrence, and overall survival.

• To characterize the tumor immune infiltrate and the expression of immune checkpoint molecules in the tumor microenvironment of colorectal cancer.

• To investigate the relationship between tumor immunological parameters and the efficacy of systemic treatments, including targeted therapies or immunotherapy.

• To integrate biological and clinical data in order to identify predictive models for disease evolution and survival.

Overall, these objectives aim to build an integrated model of prognostic assessment.

The research methodology was designed to reflect the complexity of the investigated phenomena. The study included distinct cohorts of patients, analyzed through complementary methods: clinical and surgical evaluation, histopathological analysis, molecular biology techniques, including next-generation sequencing, and advanced immunophenotyping methods. The statistical approach allowed the identification of relationships between variables and the evaluation of their impact on survival, within a rigorous and reproducible framework. (9-10)

The structure of the thesis reflects this integrative approach. The first part is dedicated to the theoretical foundations, including colorectal anatomy and physiology, mechanisms of carcinogenesis, and the role of the tumor microenvironment. The second part presents the original contributions, organized into chapters dedicated to the study objectives, methodology, results, and their interpretation. The results obtained highlight the limitations of traditional paradigms, such as the "12 lymph node rule," and support the usefulness of alternative indicators, such as the lymph node ratio. In addition, optimized protocols for liquid biopsy and tumor microenvironment analysis are presented, with potential applicability in clinical practice.

The interdisciplinary nature of the research is evident through the integration of various fields—oncological surgery, pathology, molecular biology, immunology, and medical statistics—each contributing to the construction of a unified perspective on the disease. This interdisciplinarity is not merely a methodological approach, but a necessity imposed by the complexity of colorectal cancer.

Regarding the limitations of the research, these are mainly related to the size of the analyzed cohorts and to the inherent biological variability of tumors. Moreover, the large-scale implementation of methods such as liquid biopsy or detailed analysis of the tumor microenvironment may be limited by available resources. Nevertheless, the results obtained open important perspectives for future research, aimed at validating these biomarkers in multicenter studies and integrating them into standardized clinical algorithms.

In conclusion, the present thesis aims to contribute to redefining the way survival in patients with colorectal cancer is evaluated, by proposing a modern, integrated approach oriented toward precision medicine.

General Part

The general part of the thesis consists of three major chapters and provides the theoretical foundation necessary for understanding colorectal cancer. It is structured in a progressive manner, from basic anatomical and physiological concepts to the complex mechanisms of carcinogenesis and modern diagnostic methods, including liquid biopsy in colorectal cancer.

In the first stage, the anatomy and embryology of the colon and rectum are presented in detail, highlighting structural particularities relevant to surgical and oncological practice. The segments of the colon, arterial and venous vascularization, lymphatic drainage, and innervation are described, emphasizing the importance of these elements in tumor dissemination and in the planning of surgical interventions. (11-16) Furthermore, the embryological differences between the right and left colon are analyzed and subsequently correlated with the molecular and clinical characteristics of tumors.

Subsequently, the thesis addresses colonic physiology, focusing on its essential functions: absorption of water and electrolytes, secretion, and motility. The mechanisms of ionic transport, the role of the microbiota, and the neurohormonal coordination of intestinal transit are detailed, along with the processes involved in defecation. (17-20) These aspects are relevant for understanding the symptomatology and functional alterations associated with neoplastic pathology.

A comprehensive chapter is dedicated to colorectal oncological pathology, analyzing the multistep mechanisms of carcinogenesis. The main molecular pathways involved are described—including the adenoma–carcinoma sequence, the serrated pathway, and microsatellite instability—highlighting the biological heterogeneity of the disease. (20-24) The role of genetic mutations (APC, KRAS, TP53, BRAF), chromosomal instability, epigenetic factors, as well as the influence of environmental factors and the intestinal microbiome are discussed. In this context, consensus molecular subtypes (CMS) are presented, together with their prognostic and therapeutic implications.

The thesis continues with a detailed classification of histological types of colorectal cancer, with emphasis on adenocarcinoma and its variants (mucinous, signet-ring cell, medullary, etc.), as well as other rarer tumor types. Their morphological characteristics and impact on disease evolution and therapeutic response are analyzed. (25-28)

Another important chapter is dedicated to surgical treatment, considered the gold standard in localized disease. The oncological principles of resection are described, including complete mesocolic excision and extended lymphadenectomy, as well as modern techniques such as laparoscopic and robotic surgery. Differences in the approach to colon and rectal tumors are discussed, with emphasis on total mesorectal excision and the importance of resection margins. (29-31)

Finally, the general part analyzes diagnostic methods in colorectal cancer, including colonoscopy as the reference standard, rectoscopy, histopathological confirmation, and the role of molecular assessment. The importance of adenoma detection rate, standardization of endoscopic procedures, and the integration of modern technologies such as artificial intelligence are highlighted. In addition, the role of histopathological analysis and molecular biomarkers (MSI, KRAS, NRAS, BRAF) in establishing prognosis and guiding therapy is emphasized. (32-35)

Overall, the general part constructs a coherent and comprehensive picture of colorectal cancer by integrating anatomical, physiological, molecular, and clinical data. This theoretical foundation justifies the need for a modern, multidimensional approach that goes beyond the limitations of classical assessment models and supports the research direction developed in the original part of the thesis.

Special Part

The special part presents Chapters 4–9, including the aim and objectives, the general research methodology, the three published scientific articles, as well as the conclusions and future perspectives.

Chapter 4 consists of the aim and objectives of the research, already outlined in the introduction.

Chapter 5 presents the research methodology, which was designed to capture the biological and clinical complexity of colorectal cancer through an integrative, multidisciplinary approach that combines clinical, histopathological, molecular, and immunological analyses. The study had an observational analytical design, based on the evaluation of cohorts of patients diagnosed with colorectal cancer, followed in order to identify factors influencing prognosis and survival.

Patient selection was performed based on well-defined inclusion and exclusion criteria, aiming to obtain a clinically and therapeutically homogeneous cohort. The collected data included demographic parameters, clinical characteristics, details related to surgical intervention, and information regarding administered oncological treatments. An essential component of the methodology was the assessment of surgical radicality, through the analysis of lymph node parameters, with emphasis on indicators such as the total number of examined lymph nodes and the lymph node ratio.

Histopathological analysis represented a central step, providing detailed information on tumor type, degree of differentiation, lymphovascular and perineural invasion, as well as resection margin status. These data were complemented by molecular biology investigations, including modern techniques such as next-generation sequencing, used to identify relevant mutations and the tumor molecular profile.

An innovative element of the methodology was the integration of liquid biopsy through the analysis of circulating biomarkers, with the aim of dynamically evaluating the disease. This approach allowed the correlation of molecular data with clinical evolution, treatment response, and recurrence risk. In parallel, the tumor microenvironment was characterized using immunophenotyping methods, enabling the evaluation of immune infiltrate and the expression of molecules involved in immune response regulation.

Data processing was performed using appropriate statistical methods, which allowed the identification of correlations between variables and the determination of their impact on overall survival and disease-free survival. The analyses included both descriptive and inferential methods, with predictive models being used to integrate clinical and biological data into a coherent framework.

Chapter 6: Complementing Tissue Biopsy with Plasma-Derived cfDNA NGS Analysis as Liquid Biopsy for Real-Time Therapy Modulation in Patients with Colorectal Cancer

The study investigates the role of liquid biopsy in colorectal cancer (CRC), proposing it as a complementary method to tissue biopsy for molecular characterization and real-time therapeutic adaptation. In the current context of precision medicine, therapeutic decisions are guided by the tumor molecular profile; however, conventional biopsy presents important limitations, such as its invasive nature, difficulty of repetition, and inability to reflect tumor heter-

ogeneity. Liquid biopsy, based on the analysis of circulating nucleic acids (cfDNA), offers a minimally invasive alternative capable of dynamically capturing disease evolution and identifying mutations relevant for prognosis and treatment (36,37).

The methodology included an initial cohort of 50 patients with suspected CRC, of whom 17 patients with confirmed adenocarcinoma were included in the final analysis. For each patient, both tumor samples (through NGS of genomic DNA from tissue) and peripheral blood samples (through NGS of cfDNA) were analyzed. Mutations in genes involved in colorectal carcinogenesis, such as TP53, KRAS, PIK3CA, BRAF, and others, were investigated. Bioinformatic and statistical analyses enabled comparison of mutational profiles between tissue and plasma and evaluation of correlations between molecular parameters and clinical characteristics (38).

The results demonstrated that liquid biopsy can detect a significant number of tumor mutations, with a detection rate of 38.7%. The most frequently identified mutations in both tissue and plasma were TP53 and KRAS, confirming concordance with existing literature. Furthermore, a significant correlation was observed between the allelic frequency of mutations in tissue and in plasma, although plasma values were lower. An important finding was that certain mutations were detected exclusively in plasma, suggesting the presence of tumor heterogeneity or secondary lesions not identified through tissue biopsy.

A key finding of the study is the association between mutation detection rate in plasma and the degree of tumor invasion (T stage). No mutations were identified in cases with limited tumors (T2), whereas detection rates increased significantly in advanced stages (T4), indicating that circulating tumor burden is proportional to disease extent. This finding supports the value of liquid biopsy as a tool for monitoring and evaluating tumor progression.

From a clinical perspective, the identification of relevant mutations has direct therapeutic implications. For example, KRAS and BRAF mutations influence response to anti-EGFR therapies, while PIK3CA or FBXW7 mutations may have prognostic value and open perspectives for targeted therapies. Additionally, the detection of rare mutations (such as IDH1 and MAP2K1) suggests potential directions for future research and treatment. However, the study also highlights the limitations of liquid biopsy, including the possibility of detecting mutations originating from clonal hematopoiesis, which may introduce interpretation errors (39).

In conclusion, liquid biopsy represents a promising tool in the management of colorectal cancer, providing complementary information to tissue biopsy and facilitating a personalized treatment approach. Its ability to reflect tumor heterogeneity and to monitor disease evolution in real time makes it a valuable method for prognosis, therapy selection, and early detection of therapeutic resistance. The integration of this technology into clinical practice may significantly contribute to optimizing oncological outcomes and improving patient survival.

Chapter 7: Lymph Node Yield in Colorectal Cancer: Beyond the "12-Node Rule"

The study analyzes the role of lymph node yield in the staging and prognosis of colorectal cancer, starting from the premise that lymph node status (N) is one of the most important determinants of survival. Although the threshold of a minimum of 12 examined lymph nodes is widely accepted as a quality standard, it is influenced by numerous clinical and biological factors, which limits its universal applicability. In this context, both the utility of this threshold and the role of alternative indicators, such as the lymph node ratio (LNR), are investigated in order to improve prognostic stratification (40).

The methodology consists of a retrospective observational study conducted on a cohort of 86 patients who underwent surgery for colorectal cancer. Clinical, surgical, and histopathological data were analyzed, including the total number of examined lymph nodes, the number of positive lymph nodes, the type of surgical intervention, and TNM staging. Lymph node retrieval was classified as adequate (≥12 lymph nodes) or extended (≥18 lymph nodes), and for cases with nodal involvement, the LNR was calculated. Statistical analysis included descriptive and inferential methods, as well as logistic regression models to identify factors associated with achieving the quality threshold (41).

The results show that the median number of examined lymph nodes was 12, with a compliance rate of 52.3% for the standard threshold and 31.4% for extended retrieval. Lymph node involvement was present in 25.6% of patients. Significant differences were observed according to tumor location: the right colon showed the highest lymph node yield and the highest compliance rate, while the rectum presented significantly lower values. These differences reflect anatomical particularities and the impact of neoadjuvant treatment, especially in rectal cancer. In contrast, the type of surgical approach did not significantly influence lymph node yield, suggesting that it depends more on the quality of resection and histopathological examination.

The analysis of LNR revealed a heterogeneous distribution, with 14% of patients falling into the high-risk category (LNR ≥0.20). This indicator proved particularly useful in cases with a limited number of examined lymph nodes, where it can complement the information provided by conventional staging. However, its interpretation must be performed in the context of total lymph node yield, as values may be distorted when a small number of lymph nodes are analyzed.

The discussion confirms that lymph node yield represents a composite indicator, reflecting both the quality of the surgical act and the rigor of histopathological evaluation. Data from the literature support a more flexible approach to the 12-node threshold, with target values adapted according to tumor location, stage, and therapeutic context. In addition, the introduction of LNR may significantly improve prognostic stratification, particularly in advanced stages or in situations with a risk of understaging (42-45).

In conclusion, the study supports maintaining the 12-node threshold as a minimum quality standard, while highlighting its limitations and the need for a nuanced interpretation. The integration of additional parameters, such as LNR, allows a more precise evaluation of oncological risk and may contribute to optimizing therapeutic decision-making in colorectal cancer.

Chapter 8: Optimization of a Flow Cytometry Protocol for the Detection of Immune Checkpoint Receptors PD-1/CTLA-4 in the Tumor Microenvironment of Colorectal Cancer

The study addresses the characterization of the tumor microenvironment (TME) in colorectal cancer by optimizing a flow cytometry protocol for the detection of immune checkpoint receptors PD-1 and CTLA-4. In the current context of personalized medicine, where tumor heterogeneity decisively influences therapeutic response, a detailed analysis of the interactions between tumor cells and the immune system becomes essential. The TME is composed of a complex network of cells, including tumor-infiltrating lymphocytes (TILs), tumor-associated macrophages, and neutrophils, which contribute both to tumor control and to immune evasion (46).

The study builds on the essential role of PD-1 and CTLA-4 molecules in regulating the immune response. These are expressed on T lymphocytes and act as inhibitory mechanisms, being exploited by tumor cells to evade immune destruction. In this context, immune check-

point inhibitors have revolutionized oncological treatment; however, their effectiveness depends on the tumor's immunological profile, which justifies the need for precise methods to characterize the TME.

The methodology included the collection of fresh tumor tissue from 7 patients with colorectal cancer undergoing surgical intervention. The samples were processed through enzymatic and mechanical dissociation to obtain a single-cell suspension, followed by labeling with a specific antibody panel and analysis by flow cytometry. The analysis strategy allowed the identification of relevant cellular populations (EpCam+ tumor cells, CD3+ T lymphocytes, CD4+ and CD8+ subsets) and the evaluation of PD-1 and CTLA-4 expression within these populations (47).

The results highlighted the presence of characteristic TME cellular populations, with an average of approximately 12.2% EpCam+ tumor cells and 6.8% tumor-infiltrating T lymphocytes. Among these, a significant proportion expressed checkpoint receptors: approximately 3.4% for PD-1 and 2% for CTLA-4 at the level of T cells. An important finding was the identification of receptor expression not only on immune cells but also on tumor cells, suggesting an intrinsic role of these molecules in tumor biology. These observations support the hypothesis that tumors may employ multiple mechanisms of immune evasion, not only by inhibiting lymphocytes but also by directly expressing checkpoint molecules.

The analysis confirms the utility of flow cytometry as a robust and reproducible method for TME characterization, providing both quantitative and qualitative information regarding the cellular composition and molecular profile of the tumor. In addition, this method allows the simultaneous identification of multiple cellular subpopulations, representing a major advantage over traditional techniques (48-50).

In conclusion, the study demonstrates that optimizing a flow cytometry protocol enables a detailed characterization of the tumor microenvironment in colorectal cancer, highlighting the expression of PD-1 and CTLA-4 across different cellular populations. These findings have direct implications for patient selection for immunotherapy and for the development of personalized therapeutic strategies. The integration of this approach into clinical practice may contribute to a better understanding of immune evasion mechanisms and to the optimization of oncological treatment.

9.1. Conclusions

The present thesis demonstrates that the integration of modern tumor characterization methods—lymph node yield analysis, molecular profiling through liquid biopsy, and characterization of the tumor microenvironment—allows a more accurate assessment of prognosis and an optimization of the management of patients with colorectal cancer.

The results obtained highlight that lymph node yield remains an essential element in colorectal cancer staging; however, the classical threshold of 12 lymph nodes must be interpreted within the clinical context. Tumor location, neoadjuvant treatment, and anatomopathological particularities significantly influence the number of examined lymph nodes. The integration of the lymph node ratio (LNR) provides additional prognostic information, particularly in situations with suboptimal lymph node retrieval, contributing to more accurate risk stratification and reducing the risk of understaging.

Liquid biopsy has proven to be a valuable complementary tool for the molecular characterization of colorectal cancer. The detection of mutations in plasma, consistent with the tumor profile, confirms the utility of this method in reflecting tumor heterogeneity. The correlation between detection rate and tumor size or invasiveness supports the use of liquid biopsy for disease monitoring and assessment of tumor burden, with potential in the early detection of progression or treatment resistance.

Characterization of the tumor microenvironment through flow cytometry has highlighted the complexity of immunological interactions and the relevance of the expression of immune checkpoint molecules such as PD-1 and CTLA-4. The obtained results demonstrate the feasibility of using this method for rapid and quantitative analysis of tumor and immune cell populations, providing a functional perspective on tumor immune status.

The modern methods investigated in this thesis contribute to redefining the way prognosis is assessed and how patients with colorectal cancer are managed, by overcoming the limitations of conventional approaches. Extended lymph node analysis and the use of LNR enable more precise staging and reduce the risk of disease underestimation, directly influencing the indication for adjuvant treatment. Liquid biopsy introduces a dynamic dimension into tumor characterization, allowing real-time monitoring of the molecular profile and identification of changes associated with disease progression or treatment resistance. In parallel, the evaluation of the tumor microenvironment through flow cytometry provides rapid and quantitative infor-

mation on immune status and checkpoint molecule expression, facilitating the selection of patients for immunotherapy. Through the integration of these methods, colorectal cancer management evolves from a static to a dynamic and personalized approach.

9.2. Perspectives

The extension of lymph node yield studies to larger, multicenter cohorts, with the integration of biological and immunological parameters, could allow the definition of adapted evaluation thresholds and improve prognostic stratification in patients with colorectal cancer.

In the field of liquid biopsy, future directions include the development of extended molecular panels, longitudinal dynamic analysis of samples, and the integration of these data into algorithms for monitoring minimal residual disease and therapeutic response.

Regarding the tumor microenvironment component, a major research direction is represented by the comparison of immune checkpoint molecule expression determined by flow cytometry with that assessed by immunohistochemistry, in order to validate a rapid, reproducible, and efficient protocol. Considering the longer time required for immunohistochemical evaluation, compared to flow cytometry which allows results to be obtained within 24–48 hours, this approach could have a direct clinical impact on the rapid selection of patients eligible for immunotherapy.

In the long term, the integration of histopathological, molecular, and immunological parameters into a unified predictive model could contribute to the development of personalized medicine algorithms in colorectal cancer, with an impact on survival and quality of life.

This thesis supports the transition from a static evaluation of colorectal cancer to an integrated and dynamic approach based on tumor biology. The integration of lymph node analysis, liquid biopsy, and tumor microenvironment characterization allows a more precise understanding of the disease and a more appropriate therapeutic selection. These methods have the potential to significantly improve prognosis and patient management.

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