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APPLICATIONS OF MOLECULAR BIOLOGY TECHNIQUES IN THE PERSONALIZED MANAGEMENT OF COLORECTAL CANCER

SUMMARY OF THE DOCTORAL THESIS

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Introduction

The title of the doctoral thesis, **"Applications of Molecular Biology Techniques in the Personalized Management of Colorectal Cancer",** reflects current concerns in the field of digestive oncology regarding the integration of molecular biomarkers into diagnosis, prognostic assessment, and the optimization of therapeutic strategies for patients with malignant diseases. Colorectal cancer (CRC) represents one of the most important public health challenges, both because of its high incidence and mortality and because of its clinical and biological heterogeneity. In Romania, CRC is the most frequently diagnosed cancer in both sexes and ranks second among causes of cancer-related mortality, which justifies the growing interest in the development of modern tools useful for the diagnostic and therapeutic management of this disease [1]. In this context, clinical, imaging, and histopathological evaluation is not always sufficient to capture the biological diversity of colorectal tumors, which supports the need for the use of modern molecular biology techniques.

The motivation for choosing this research topic derived from the need to overcome the limitations of the conventional approach to CRC through the integration of molecular biomarkers into the personalized management of these patients. Differences in biological aggressiveness, therapeutic response, and prognosis observed in patients with the same tumor stage suggest the existence of important molecular particularities that remain insufficiently exploited in current medical practice. The relevance of the topic is further supported by the increasing incidence of early-onset colorectal cancer (EO-CRC), defined as CRC diagnosed at an age of ≤50 years, as well as by the expanding use of molecular biomarkers and the growing interest in non-invasive tests and personalized medicine. Thus, although the implementation of screening programs has improved the early diagnosis of CRC in patients over 50 years of age and has contributed to reducing the global burden associated with this disease, the proportion of patients with EO-CRC has steadily increased over recent decades [2–4]. From a molecular perspective, compared with CRC diagnosed after the age of 50 years, namely late-onset colorectal cancer (LO-CRC), EO-CRC exhibits a different frequency of oncogenic mutations, a higher prevalence of mucinous histology and poor differentiation, a distinct DNA methylation profile, a more distal tumor location, and lower survival rates [2,5]. In line with these data, in

2018 the American Cancer Society lowered the recommended age for initiating CRC screening strategies to 45 years [6]. In Europe, however, guidelines still recommend the implementation of screening strategies in individuals aged between 50 and 74 years [7]. Recent data also suggest an increasing trend in the incidence of EO-CRC at the European level [8,9].

On the other hand, genomic, epigenomic, transcriptomic, and proteomic biomarkers have proven to represent an essential direction for optimizing the management of patients with CRC. To date, the Food and Drug Administration (FDA) has approved several molecular tests for the non-invasive screening of CRC, including Cologuard, Cologuard Plus, ColoSense, Shield, and ColoHealth [6,10]. In addition to their contribution to the early detection of disease, these biomarkers may provide valuable information regarding prognosis, the risk of tumor progression, the probability of recurrence, and response to oncologic therapies. Thus, the identification of molecular alterations such as KRAS, NRAS, and BRAF mutations, mismatch repair deficiency/microsatellite instability (MMR/MSI) status, amplification of human epidermal growth factor receptor 2 (HER2), or transcriptomic and proteomic changes associated with chemoresistance enables a more precise biological stratification of patients and supports the selection of a personalized therapeutic approach. In this context, molecular tests do not have solely diagnostic value, but also represent tools of predictive utility, contributing to treatment individualization and, consequently, to the improvement of patient prognosis in CRC.

The doctoral thesis is structured into two main sections: the general part and the section dedicated to personal contributions. The general part comprises three chapters presenting epidemiological and etiopathogenic data, aspects related to the diagnosis and screening of CRC, and the role of molecular biomarkers in the management of this disease. The section of personal contributions includes seven chapters presenting the working hypothesis and general objectives of the research, the research methodology, and the results of the studies conducted throughout the doctoral project. The content of the thesis, resulting both from the critical analysis of the literature and from the original research undertaken, was disseminated through the publication of 19 original articles and review papers in internationally indexed journals. Overall, the structure of the thesis integrates the theoretical background of the topic with the findings of the doctoral research on CRC.

Personal Contributions

Chapter 4. Working Hypothesis and General Objectives of the Research

The research hypothesis of the thesis was that the clinical and biological heterogeneity of CRC can be more accurately characterized through the integration of molecular biology techniques with clinical, histopathological, and immunohistochemical data, and that this approach may lead to the identification of biomarkers useful for early diagnosis, prognostic stratification, and the personalization of therapeutic management. In accordance with this hypothesis, the main objective of the research was to investigate the role of modern molecular biology techniques in the characterization of CRC and in the optimization of patient management through the integrated analysis of molecular, immunohistochemical, and inflammatory markers, as well as their correlations with the clinicopathological features of the disease. The specific objectives of the doctoral research included:

1. The comparative evaluation of epidemiological, clinical, imaging, histopathological, and immunohistochemical characteristics in patients with EO-CRC compared with those with LO-CRC, in order to identify factors associated with the more aggressive course of EO-CRC. In addition, the study aimed to provide a more comprehensive characterization of EO-CRC in the Romanian population and to identify elements useful for improving diagnostic strategies and risk stratification.
2. The evaluation of MMR status in patients with CRC and the analysis of correlations between MMR deficiency and clinical, histopathological, immunohistochemical, and inflammatory characteristics, with the aim of identifying markers with prognostic relevance and potential utility in guiding therapeutic management.
3. The evaluation of the plasma profile of inflammatory mediators involved in colorectal carcinogenesis (TNF-R, total TNF-α, PDGF-AA, IL-17A, and IL-1β) in patients with colorectal polyps, EO-CRC, and LO-CRC, compared with healthy individuals, in order to identify inflammatory signatures with diagnostic and prognostic potential.
4. The evaluation of the expression profile of selected HERV-associated transcripts in colorectal tumor tissue compared with adjacent non-tumoral tissue, and the investigation of their relationship with molecular biomarkers involved in inflammation, cell proliferation

and cell-cycle control, adaptation to the hypoxic tumor microenvironment, angiogenesis, epithelial integrity, and tumor invasion, with the aim of clarifying their biological relevance and their potential as diagnostic and prognostic biomarkers, as well as potential therapeutic targets.

Chapter 5. General Research Methodology

The doctoral research had a mixed design, including two retrospective studies and two prospective studies, all of an exploratory nature. These studies enrolled patients from the Bucharest Emergency Clinical Hospital with histopathologically confirmed CRC over a three-year period, between February 2023 and February 2026, as follows:

- The first study included 204 patients with CRC, who were divided into two groups: Group 1 – patients with EO-CRC and Group 2 – patients with LO-CRC;
- The second study included 264 patients with CRC, who were divided into two groups: Group 1 – patients with positive MMR status and Group 2 – patients with negative MMR status;
- The third study included 51 patients with LO-CRC, 11 patients with EO-CRC, 16 patients with colorectal polyps, and 10 healthy subjects without known colorectal lesions, from whom peripheral blood samples were collected;
- The fourth study included 21 patients with CRC, from whom tissue samples and peripheral blood samples were collected.

Prior to the initiation of the research, approval was obtained from the Ethics Committee of the Bucharest Emergency Clinical Hospital (approval no. 1400/07.02.2023), and, for the processing of biological samples at the Ștefan S. Nicolau Institute of Virology in Bucharest, a framework collaboration agreement was concluded between the two institutions (no. 560/12.04.2022). Patients included in the retrospective studies had provided consent for the use of their medical data for research purposes, while those enrolled in the prospective studies additionally signed an informed consent form for the collection of biological samples.

The general inclusion criteria consisted of histopathologically confirmed CRC diagnosis and signed informed consent, to which study-specific criteria were added, such as the availability of immunohistochemical results or the absence of cancers of other sites, recent

infections, and autoimmune diseases. The exclusion criteria included refusal to participate in the study, the presence of synchronous malignancies, recent infections or autoimmune diseases, and the absence of relevant clinical or paraclinical data required for statistical analysis.

The research methodology comprised several successive stages: defining the theoretical framework through analysis of the scientific literature, formulating the hypothesis and objectives, developing the study protocol, establishing the patient cohorts, and standardizing the procedures for the collection, transport, processing, and storage of biological samples. Demographic, clinical, and paraclinical data were collected from medical records and centralized in dedicated databases. For the prospective studies, peripheral blood samples and, where applicable, paired tumor tissue and adjacent non-tumoral tissue samples were collected. Blood samples were centrifuged, plasma was separated and stored at −80°C, while tissue samples were obtained from surgical specimens, transported under standardized conditions, and preserved under the same conditions until analysis.

Plasma cytokine concentrations were determined using the enzyme-linked immunosorbent assay (ELISA) method, while tissue gene expression analysis was performed by quantitative real-time polymerase chain reaction (qRT-PCR) following total RNA extraction and reverse transcription. Thus, the plasma concentrations of several cytokines with a well-established role in oncogenesis were determined, namely: soluble tumor necrosis factor receptor superfamily members (sTNF-R), total tumor necrosis factor alpha (total TNF-α), platelet-derived growth factor AA (PDGF-AA), interleukin 17A (IL-17A), and interleukin 1β (IL-1β), according to established protocols. In addition, tissue expression was assessed for a series of transcripts associated with human endogenous retroviral elements (HERV) (HERV-FRD, HERV-R, HERV-V1, HERV-1, HERV-H, HERV-K, endogenous retroviral family W, ENV member 1 – ERVWE1), immunomodulatory molecules such as human endogenous retrovirus-H long terminal repeat-associating 1 (HHLA1) and human endogenous retrovirus-H long terminal repeat-associating 2 (HHLA2), the interleukin 17 family (IL-17A–F), and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB). Subsequently, an extended panel of markers involved in cell proliferation, angiogenesis, cell adhesion, and epithelial barrier integrity was evaluated, including dihydrofolate reductase (DHFR), proliferating cell nuclear

antigen (PCNA), Kiel 67 antigen (Ki67), tyrosine kinase with immunoglobulin-like and EGF-like domains (TIE1), cyclin B1 (CCNB1), angiopoietin 1 (ANGP1), occludin (OCLN), tight junction protein 1 (TJP1), Egl-9 family hypoxia-inducible factor 2 (EGLN2), tissue inhibitor of metalloproteinase 1 (TIMP1), CD62 (L-selectin), beta-catenin, intercellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM), epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor 2 (VEGFR2), and endothelin 1 (EDN1). Relative expression levels were calculated using the comparative Ct method (2^-ΔΔCt), normalized to the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Statistical analysis was performed using SPSS, R, and Python, depending on the objectives of each study. Finally, the results were integrated in order to outline a specific profile of colorectal carcinogenesis and to identify biomarkers with diagnostic, prognostic, and therapeutic potential. Statistical significance was set at $p < 0.05$.

Chapter 6. Prognostic differences between early-onset and late-onset colorectal cancer

Introduction. Studies conducted in recent years have reported an increasing incidence of EO-CRC, which appears to represent a distinct entity compared with LO-CRC, owing to its epidemiological, anatomical, histopathological, and molecular particularities [11]. Moreover, recent research estimates that by 2030, the rates of diagnosis of early-onset colon cancer will increase by 27.7%, while those of early-onset rectal cancer will rise by 46% [12]. Unfortunately, the alarming increase in the incidence of EO-CRC described by epidemiological studies has not been accompanied by a substantial number of observational or experimental studies capable of improving both the early diagnosis of this condition and the optimization of therapeutic management [13]. In this context, the objective of our study was the comparative evaluation of demographic, clinical, histopathological, and immunohistochemical variables in patients with EO-CRC and those with LO-CRC, in order to identify features that might explain the more aggressive behavior of EO-CRC and contribute to improving the management of these patients [11].

Materials and Methods. We conducted a retrospective, observational study over a two-year period (January 2021–January 2023), which included 204 patients admitted to the Bucharest Emergency Clinical Hospital with a histopathologically confirmed diagnosis of CRC. Patients were divided into two groups according to age at diagnosis: the EO-CRC group, comprising patients diagnosed with CRC at the age of 50 years or younger, and the LO-CRC group, comprising patients diagnosed after the age of 50 years [11].

Demographic, clinical, endoscopic, histopathological, and immunohistochemical variables were analyzed, together with comorbidities, family history, serum values of selected biological markers, and length of hospital stay. Statistical data processing was performed using IBM SPSS Statistics, applying comparative tests for categorical and continuous variables, as well as linear and logistic regression models to assess independent associations and control for potential confounding factors [11].

Results, Discussion, and Conclusions. Of the total number of patients included in the study, 11.3% were diagnosed with EO-CRC, most of whom were in the 45–50-year age group (61%) [11]. These findings support data from the literature suggesting that CRC screening in the 45–50-year age group could have a significant impact on the prognosis of these patients [11,14]. A slight predominance of male sex was also observed in both study groups, although it was more pronounced among patients with EO-CRC (64.4% versus 53.6%), without reaching statistical significance ($p = 0.509$) [11].

Patients with EO-CRC more frequently presented distally located tumors and a stenosing endoscopic appearance (43.5% versus 29.3%), although this difference did not reach statistical significance [11]. We also evaluated TNM staging and identified a tendency toward diagnosis at advanced stages in both patient groups [11]. By contrast, from a histopathological perspective, mucinous adenocarcinoma was more frequent in patients with EO-CRC than in those with LO-CRC (34.8% versus 14.4%) [11]. Similarly, these patients showed a higher proportion of poorly differentiated tumors (39.1% versus 14.2%; $p = 0.010$), findings that support the more aggressive nature of this disease subtype [11].

Immunohistochemical results, available in a limited number of patients, revealed statistically significant differences in the expression of caudal-type homeobox transcription

factor 2 (CDX2) and HER2 between the two study groups. Thus, CDX2 was negative in all evaluated EO-CRC patients, whereas 58.5% of LO-CRC patients showed positive CDX2 expression ($p = 0.012$). In contrast, HER2 was positive in 50% of EO-CRC patients and in none of the LO-CRC patients ($p = 0.002$) [11]. In addition, the positivity rate of mutS homolog 2 (MSH2) was higher in the LO-CRC group (76.5% versus 33.3%), a difference that approached the threshold of statistical significance ($p = 0.056$) [11]. HER2 overexpression in EO-CRC may contribute to tumor aggressiveness by activating key oncogenic pathways, including PI3K/AKT and MAPK signaling, which promote cell proliferation, survival, and metastasis [15,16]. Furthermore, HER2 alterations have been associated with resistance to conventional chemotherapy, suggesting a potential benefit of anti-HER2 targeted therapies in selected patients with EO-CRC [17].

Logistic regression analysis identified smoking and hypertension as factors independently associated with EO-CRC. For smoking, the odds ratio (OR) was 3.450, indicating a 3.45-fold higher risk of EO-CRC in smokers compared with non-smokers [11]. Regarding the association between hypertension and the increased risk of EO-CRC, we consider that this finding more likely reflects hypertension as part of the metabolic syndrome [11]. Concerning biological markers, no significant differences were identified for carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA 19-9), whereas serum albumin levels were significantly higher in patients with EO-CRC ($p = 0.002$) [11]. The mean duration of hospitalization for patients included in our study was 14.51 days, and the only parameter that significantly influenced hospital stay was the serum albumin level [11].

In conclusion, the results of our study support the more aggressive nature of EO-CRC compared with LO-CRC, as reflected by the higher frequency of mucinous histology, poor tumor differentiation, increased expression of pro-oncogenic factors such as HER2, and reduced expression of tumor suppressor factors such as CDX2 [11]. At the same time, the association with modifiable risk factors, such as smoking and hypertension, underscores the need for preventive strategies and personalized screening approaches [11].

Chapter 7. Clinical, immunohistochemical, and inflammatory profile in

colorectal cancer: the impact of MMR status

Introduction. The evaluation of mismatch repair (MMR) status has proven to play an important role in the management of patients with CRC, given its diagnostic, prognostic, and therapeutic implications [18]. MMR deficiency leads to microsatellite instability (MSI) and is frequently associated with certain clinicopathological features, such as proximal tumor location, advanced tumor stage, poor differentiation, and abundant tumor-infiltrating lymphocytes [19]. However, the prognostic role of MSI in the assessment of patients with CRC remains controversial. In this context, we aimed to evaluate MMR status in patients with CRC, as well as to identify correlations between their clinical, immunohistochemical, and inflammatory profiles [18]. The secondary objective was to identify potential prognostic biomarkers that may guide the therapeutic management of CRC in order to improve both the quality and duration of life of these patients [18].

Materials and Methods. We conducted a retrospective, observational study that included 264 patients with histopathologically confirmed CRC who were admitted to the Bucharest Emergency Clinical Hospital over a 10-year period (January 2015–January 2025). Patients were divided into two groups according to MMR status: the group with preserved expression of all MMR proteins (MMR-positive status) and the group with loss of expression of at least one of the following proteins: mutL homolog 1 (MLH1), MSH2, mutS homolog 6 (MSH6), or postmeiotic segregation increased 2 (PMS2) (MMR-negative status). Clinical, histopathological, immunohistochemical, and inflammatory variables were analyzed, including tumor location, degree of differentiation, TNM stage, the expression of markers such as CDX2, cytokeratin 7 (CK7), cytokeratin 20 (CK20), and Ki67, as well as the systemic inflammatory markers NLR (neutrophil-to-lymphocyte ratio) and PLR (platelet-to-lymphocyte ratio) [18]. Statistical analysis was performed using Python, applying comparative tests, regression models, and SHAP value interpretation to assess the impact of predictors on clinical outcomes [18].

Results, Discussion, and Conclusions. MMR deficiency was identified in 18.18% of patients. Among those with MMR-negative status, 6.25% showed loss of expression of a single protein, 91.67% showed loss of expression of two proteins, and 2.08% (a single patient) showed loss of expression of all four proteins [18]. The most frequent alteration was the loss of MLH1

expression, followed by PMS2, while the concurrent loss of MLH1/PMS2 expression was observed in 77.08% of patients with MMR deficiency [18]. Regarding the Ki67 proliferation index, evaluated in 39% of patients, 97.08% had values above the 25% threshold [18]. Comparative analysis showed that MMR deficiency was more frequent in women ($p < 0.001$), in patients with right-sided colon tumors ($p < 0.001$), and in those with poorly differentiated tumors (62.5% G3 in the MMR-negative group; $p < 0.001$) [18]. In addition, loss of CDX2 expression was significantly more frequently associated with MMR deficiency (42.85% versus 5.36%; $p < 0.001$) [18]. By contrast, the systemic inflammatory markers NLR and PLR did not show statistically significant differences between groups.

The individual analysis of MMR proteins revealed distinct associations. Thus, loss of MLH1 expression was more frequent in women ($p = 0.004$), in patients with right-sided colon tumors ($p < 0.001$), and in those with poorly differentiated tumors ($p < 0.001$) [18]. Furthermore, loss of MLH1 expression was associated with loss of CDX2 expression ($p < 0.001$) [18]. Loss of MSH2 expression was rare (≤3.6%) and was significantly correlated only with poorly differentiated tumors ($p = 0.01$) [18]. Similarly, loss of MSH6 expression, observed in fewer than 3% of patients, was significantly associated with histological type, being identified in 20% of signet-ring cell adenocarcinomas, 6.5% of mucinous adenocarcinomas, 0.8% of adenocarcinomas NOS, and 0% of neuroendocrine tumors ($p = 0.001$) [18]. Loss of PMS2 expression was significantly associated with female sex ($p = 0.015$), right-sided colon location ($p = 0.003$), and poor tumor differentiation ($p < 0.001$) [18].

The mean length of hospital stay was 13.9 days, and the in-hospital mortality rate was 7.69% [18]. Multivariate regression analyses and Random Forest models, complemented by SHAP value interpretation, showed that the main factors associated with prolonged hospitalization were MMR-positive status and poor tumor differentiation, while right-sided colon location had a more modest effect [18]. The strongest predictor of in-hospital mortality was mucinous adenocarcinoma, followed by right-sided colon location and poor tumor differentiation [18]. With regard to overall survival, no statistically significant differences were identified between patients with MMR-positive status and those with MMR deficiency, as the death rate was similar between the two groups (35.6% versus 35.4%, 95% CI 0.51–1.90) [18].

However, SHAP analysis suggested that poor tumor differentiation and right-sided colon location were associated with an increased risk of all-cause mortality, whereas MMR-positive status had a modest but generally unfavorable effect [18].

In conclusion, our results support the finding that MMR deficiency is associated with a distinct clinicopathological profile, characterized by younger age, female sex, proximal tumor location, poor differentiation, and loss of CDX2 expression, thereby confirming the value of MMR status as a relevant biomarker for the biological characterization and prognostic stratification of patients with CRC [18].

Chapter 8. Plasma expression of sTNF-R, TNF-α, PDGF-AA, IL-17A, and IL-1β as potential biomarkers in colorectal cancer

Introduction. Studies conducted in recent years have focused both on elucidating the pathophysiological mechanisms leading to the development of CRC and on identifying novel tools capable of facilitating early diagnosis and optimizing the therapeutic management of these patients. In this regard, the accessibility and non-invasive nature of blood-based tests have generated considerable interest [20–23]. Moreover, among the various mechanisms involved in carcinogenesis, the inflammatory pathway has attracted particular attention, with numerous studies describing the cellular and molecular roles of inflammation in tumor initiation and progression [23–25]. Nevertheless, the exact mechanisms by which chronic inflammation contributes to carcinogenesis have not yet been fully elucidated [26].

In this context, the present study aimed to investigate the relationship between systemic inflammatory mediators and CRC by analyzing the plasma levels of five cytokines with well-established roles in tumor biology: sTNF-R, total TNF-α, PDGF-AA, IL-17A, and IL-1β [27]. These cytokines were selected because they represent essential and complementary components of pro-oncogenic inflammatory signaling: IL-1β and TNF-α are central activators of the NF-κB/STAT3 pathways, sTNF-R is a stable systemic indicator of TNF-mediated inflammation, PDGF-AA reflects stromal activation and angiogenesis, while IL-17A captures the T helper 17 (Th17) axis implicated in tumor invasion [27]. The originality of this study lies in the comparative evaluation of these biomarkers across four distinct groups: healthy controls,

patients with colorectal polyps, patients with EO-CRC, and patients with LO-CRC [27]. The primary objective was to identify expression differences capable of distinguishing between stages of colorectal carcinogenesis, while the secondary objective was to evaluate the combined potential of these cytokines for the development of a diagnostic kit [27].

Materials and Methods. We conducted an exploratory, prospective, case-control study between February 2023 and October 2025, including 10 healthy subjects, 16 patients with colorectal adenomatous polyps, 11 patients with EO-CRC, and 51 patients with LO-CRC [27]. All participants provided informed consent regarding biological sample collection and the use of medical data for research purposes, and the study was approved by the Ethics Committee of the Bucharest Emergency Clinical Hospital. Peripheral blood samples were collected from each subject and transported to the Ștefan S. Nicolau Institute of Virology in Bucharest for processing and storage. Following centrifugation, plasma was stored at −80°C until analysis. Plasma concentrations of sTNF-R, total TNF-α, PDGF-AA, IL-17A, and IL-1β were determined using ELISA, with validated commercial kits. Statistical analysis was performed using R and Python, applying non-parametric tests, adjustment for multiple testing by the Benjamini–Hochberg method (FDR), effect size estimation, ROC analyses, and multivariable logistic regression models [27].

Results, Discussion, and Conclusions. Descriptive analysis identified statistically significant differences among the pathological groups with respect to age, smoking status, tumor location, and tumor stage [27]. The group of patients with colorectal polyps had the highest proportion of smokers (75%), followed by the EO-CRC group (63.6%) and subsequently the LO-CRC group (33.3%) [27]. In addition, patients with colorectal polyps more frequently presented lesions located in the left colon, patients with EO-CRC showed a predominantly distal distribution of tumor lesions, whereas those with LO-CRC more frequently exhibited right-sided colon tumors ($p = 0.010$) [27]. In terms of tumor stage, patients with EO-CRC were more frequently diagnosed with stage IV disease compared with those with LO-CRC (45.5% versus 15.7%; OR = 4.48; $p = 0.043$) [27].

Among the five cytokines analyzed, PDGF-AA showed the most marked differences between the control group and all pathological groups, with substantially higher values in

patients with colorectal polyps and CRC compared with healthy subjects [27]. After adjustment for multiple testing, the differences in plasma PDGF-AA levels between the analyzed groups remained statistically significant, confirming this biomarker as the most robust among those investigated. By contrast, sTNF-R showed higher levels in control subjects than in pathological groups, but these differences did not remain statistically significant after FDR correction [27]. No statistically significant differences were identified between groups for total TNF-α and IL-1β [27]. IL-17A showed modest differences: control subjects had slightly higher levels than patients with LO-CRC, and patients with EO-CRC had slightly higher levels than those with LO-CRC, although these differences did not retain statistical significance after FDR adjustment [27].

The evaluation of the diagnostic performance of the five-cytokine panel demonstrated a good apparent discriminative capacity between healthy subjects and pathological groups, with AUC values ranging from 0.90 to 0.99. In contrast, the capacity to differentiate between pathological subgroups was modest, with AUC values ranging from 0.60 to 0.66 [27]. A reduced panel consisting of sTNF-R, PDGF-AA, and IL-17A also showed good apparent discriminative ability between healthy subjects and pathological groups; however, after internal validation by bootstrap, the corrected AUC values decreased, and comparisons between pathological subgroups likewise revealed only modest discriminative capacity [27].

In conclusion, our results suggest that systemic inflammatory signaling may reflect certain aspects of colorectal oncogenesis. PDGF-AA emerged as the most promising plasma biomarker among those analyzed for distinguishing healthy subjects from patients with colorectal lesions, in line with its recognized role in stromal activation and angiogenic signaling [27]. Both the extended five-cytokine multimarker panel and the reduced panel consisting of the cytokines that showed the largest differences between the analyzed groups demonstrated good apparent discriminative capacity between healthy subjects and patients with colorectal lesions, but only modest accuracy in differentiating pathological subgroups from one another. Further studies in larger cohorts are needed to validate our findings.

Chapter 9. Differential expression of selected HERV transcripts and their correlations with molecular biomarkers in colorectal cancer

Introduction. Studies conducted in recent years have focused on the etiopathogenesis of CRC, with particular attention being paid to the role of the intestinal microbiome, as well as that of HERVs [28–30]. HERVs account for approximately 8% of the human genome [31]. The relationship between HERV expression and oncogenesis remains controversial and may be explained either by epigenetic dysregulation or by their promoter activity on oncogenes [32]. The mechanisms through which HERVs may influence protein synthesis are multiple and include the direct production of proteins derived from internal HERV sequences (Env, Gag, Pol), the phenomenon of oncoexaptation between a gene and a HERV element, and the action of HERVs as long non-coding RNAs (lncRNAs) capable of modulating gene expression [32,33]. In light of these considerations, the aim of the present study was to evaluate the expression profile of selected HERV transcripts both in colorectal tumor tissue and in adjacent non-tumoral tissue and to explore their relationships with an extended panel of biomarkers involved in key processes of oncogenesis. We selected the transcripts HERV-FRD, HERV-R, HERV-K, HERV-V1, HERV-1, HERV-H, and ERVWE1, as well as the immunoregulatory molecules HHLA1 and HHLA2, because of their biological relevance in carcinogenesis, including the potential of certain members of this group to generate Env-type proteins, to be associated with epigenetic dysregulation, and to contribute to viral mimicry, interferon activation, and remodeling of the tumor immune context. In parallel, we selected biomarkers covering complementary dimensions of CRC biology: tumor inflammation and immune signaling (IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, IL-17F, NFκB), cell proliferation and cell-cycle control (Ki67, PCNA, DHFR, CCNB1, EGFR), angiogenesis and adaptation to the hypoxic tumor microenvironment (TIE1, VEGFR2, ANGP1, EGLN2, EDN1), and invasion, metastasis, and epithelial–mesenchymal transition (adhesion/invasion markers—ICAM, VCAM, CD62/L-selectin, TIMP1; epithelial integrity markers—beta-catenin, OCLN, TJP1). More specifically, we aimed to characterize the co-expression patterns between HERV families and these biomarkers and to assess whether their variations align with molecular signatures suggestive of a specific tumor microenvironment. Through this integrative approach, our study

sought to clarify the biological significance of HERV expression in CRC and to explore their potential as diagnostic, prognostic, and therapeutic molecular biomarkers. Furthermore, given the very limited data regarding the involvement of HERVs in colorectal carcinogenesis, we also aimed to open new avenues for future research.

Materials and Methods. We conducted an exploratory, single-center study that included 21 patients with CRC who underwent surgery with either curative or palliative intent and who had not received prior oncologic treatment. The study was carried out between February 2023 and February 2025 at the Bucharest Emergency Clinical Hospital and the Ștefan S. Nicolau Institute of Virology in Bucharest. All participants signed informed consent forms, and the study was approved by the Ethics Committee of the Bucharest Emergency Clinical Hospital.

Paired samples of tumor tissue and adjacent non-tumoral tissue were collected from each patient from surgical resection specimens before formalin fixation. Following transport and storage at −80°C, total RNA was extracted using TRIzol reagent, followed by assessment of RNA purity and integrity. Subsequently, cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit, and target gene expression was analyzed by quantitative real-time PCR using Maxima SYBR Green qPCR Master Mix and primers validated by OriGene Technologies. Relative gene expression was calculated using the Ct method (2^-ΔΔCt), normalized to the reference gene GAPDH. Statistical analysis was performed using R, applying non-parametric tests for paired samples, the Wilcoxon signed-rank test, the exact McNemar test, Spearman correlations for paired differential expression Δ(T−N), and the Benjamini–Hochberg correction to control type I error.

Results, Discussion, and Conclusions. The study cohort included 21 patients, with a relatively balanced sex distribution and a mean age of 64.2 years. Tumors were more frequently located in the left colon (42.9%), while right-sided colon and rectal tumors had comparable proportions (28.6% each). Histologically, adenocarcinoma NOS predominated (90.5%), and most tumors were moderately differentiated (81% G2). Stage II was the most frequent stage, and among comorbidities, hypertension (52.4%) and diabetes mellitus (38.1%) were the most prevalent.

Paired analysis of HERV transcript expression revealed a selective pattern of dysregulation at the tumor level. HERV-FRD, HERV-R, HERV-K, HERV-V1, ERVWE1, HHLA1, and HHLA2 showed significant overexpression in tumor tissue compared with adjacent non-tumoral tissue ($p \leq 0.031$). In contrast, HERV-1 and HERV-H did not show consistent differences between tumor and adjacent non-tumoral tissue. With regard to inflammatory and immunological biomarkers, IL-17E was the only cytokine of the IL-17 family that showed a significant increase in expression in tumor tissue ($p = 0.001$). IL-17C showed a trend toward tumor overexpression, but without statistical significance. For IL-17A, IL-17B, and IL-17F, the low number of quantifiable samples limited inferential analysis. Nevertheless, IL-17A showed significantly greater detectability in tumor tissue than in adjacent non-tumoral tissue (McNemar $p = 0.031$). NFκB did not show statistically significant differences between the two tissue types.

Analysis of proliferation and cellular metabolism markers demonstrated significant tumor overexpression of Ki67, PCNA, and DHFR, all with statistically significant p values, supporting the existence of an active proliferative phenotype at the tumor level. By contrast, CCNB1 showed a negative paired median difference, suggesting higher expression in adjacent non-tumoral tissue ($p = 0.014$), while EGFR showed a slight trend toward reduced expression in tumor tissue, without statistical significance. Among the markers involved in angiogenesis and adaptation to hypoxia, TIE1 was detected exclusively in tumor tissue and approached the threshold of statistical significance, VEGFR2 had greater detectability in tumor tissue (McNemar $p = 0.049$), and EGLN2 was significantly overexpressed in tumor tissue ($p = 0.030$). EDN1 showed only a trend toward increased expression in tumor tissue, without statistical significance. Regarding adhesion and epithelial integrity markers, ICAM, VCAM, and TIMP1 showed modest increases in tumor expression without statistical significance. In contrast, beta-catenin was significantly underexpressed in tumor tissue ($p = 0.024$), while OCLN showed a trend toward reduced tumor expression.

Spearman correlation analysis based on paired differential expression $\Delta(T-N)$ revealed a coherent pattern of covariation among several HERV transcripts (HERV-FRD, HERV-K, HERV-V1, HERV-R, ERVWE1), suggesting possible coordinated dysregulation of their

expression in the tumor context. Moreover, tumor-overexpressed HERV transcripts correlated positively with IL-17E, suggesting the association of this HERV/ERVWE1 subset with an inflammatory microenvironment linked to the IL-17E axis, in the absence of significant correlations with NFκB. Likewise, HERV-FRD, HERV-K, HERV-V1, HERV-R, ERVWE1, and HHLA2 showed significant positive correlations with the proliferation markers Ki67, PCNA, and DHFR, supporting the integration of HERV reactivation into an active proliferative-metabolic program. In addition, among the markers involved in adaptation to the hypoxic microenvironment, EGLN2 showed consistent positive correlations with tumor-overexpressed HERV transcripts, suggesting a link between their reactivation and the hypoxic tumor response. With regard to markers of invasion and tissue remodeling, TIMP1 correlated with HERV-FRD, HERV-K, HERV-V1, and HERV-R, while ERVWE1 showed significant positive correlations with ICAM and VCAM. In contrast, these HERV transcripts showed significant negative correlations with beta-catenin and OCLN, supporting the hypothesis of a link between HERV reactivation, loss of epithelial integrity, and activation of epithelial–mesenchymal transition processes.

No robust associations were identified between HERV transcript expression and tumor location, histological type, tumor stage, or degree of differentiation, with most comparisons showing small effect sizes and statistically non-significant p values. However, these findings must be interpreted in the context of the small sample size and the relatively uneven distribution of clinicopathological characteristics.

In conclusion, our study identified a selective profile of HERV transcript reactivation in CRC, characterized by tumor overexpression of HERV-FRD, HERV-R, HERV-K, HERV-V1, ERVWE1, HHLA1, and HHLA2. The integration of these data with the expression of an extended biomarker panel suggests that HERV reactivation does not represent an isolated phenomenon, but rather is associated with biologically relevant axes such as IL-17E-type tumor inflammation, cell proliferation and metabolism, adaptation to hypoxia, tissue remodeling, immune evasion, and altered epithelial integrity. Our results support the hypothesis that selective HERV reactivation may represent a biologically relevant element in colorectal

carcinogenesis and provide a basis for future studies aimed at mechanistic validation and the exploration of these transcripts as diagnostic, prognostic, or potential therapeutic biomarkers.

Chapter 10. Conclusions and personal contributions

The main objective of the present doctoral thesis was to investigate the role of molecular biology techniques in the characterization of CRC and in the optimization of patient management. Accordingly, we performed an integrated analysis of the epidemiological, clinical, histopathological, immunohistochemical, proteomic, and transcriptomic particularities of the disease. The results obtained demonstrate that the proposed research objectives were achieved and support the usefulness of a multimodal approach for understanding the heterogeneity of CRC. The personal contributions of this thesis include the following:

1. A comparative characterization of EO-CRC versus LO-CRC in a Romanian patient cohort, highlighting clinicopathological and immunohistochemical differences suggestive of a more aggressive biological behavior of EO-CRC. Thus, we demonstrated the association of EO-CRC with a higher proportion of poorly differentiated tumors, mucinous histology, increased HER2 expression, and reduced CDX2 expression, features that contribute to defining a distinct biological signature for this patient subgroup.
2. The performance of a clinical, immunohistochemical, and inflammatory analysis in relation to MMR status in a Romanian cohort of patients with CRC, demonstrating the significant association of MMR deficiency with female sex, proximal tumor location, poor differentiation, and loss of CDX2 expression.
3. A separate analysis of the loss of expression of each of the proteins MLH1, MSH2, MSH6, and PMS2, and of their relationship with clinicopathological parameters, which represents an original contribution with diagnostic and prognostic relevance.
4. The demonstration of the role of PDGF-AA as a plasma inflammatory biomarker with good ability to differentiate healthy individuals from patients with benign or malignant colorectal lesions, together with the comparative evaluation of cytokine panels with exploratory potential for the non-invasive screening of CRC.

5. The identification of a selective profile of tumor HERV transcript reactivation in CRC, characterized by the overexpression of HERV-FRD, HERV-R, HERV-K, HERV-V1, ERVWE1, HHLA1, and HHLA2, thereby contributing to the definition of a new biological framework for colorectal carcinogenesis.
6. The demonstration of significant correlations between tumor-overexpressed HERV transcripts and biomarkers involved in inflammation (IL-17E), proliferation and cellular metabolism (Ki67, PCNA, DHFR), hypoxia (EGLN2), tissue remodeling, and impaired epithelial integrity (TIMP1, ICAM, VCAM, beta-catenin, OCLN), thereby generating original hypotheses regarding the association of HERV reactivation with a particular biological profile.
7. The integration, in a unified manner, of epidemiological, clinical, histopathological, immunohistochemical, proteomic, and transcriptomic data, contributing to the standardization of the methodological stages of prospective studies. At the same time, we formulated concrete future research directions for the development of multimarker panels useful for the diagnostic evaluation, prognostic stratification, and personalization of treatment in CRC.

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