Summary

I. Current State of Knowledge

Colorectal cancer is among the most common malignancies worldwide, representing the second leading cause of cancer-related mortality. Its incidence is influenced by genetic, epigenetic, inflammatory, and environmental factors, which makes early diagnosis and therapeutic management particularly challenging. Recent advances in biomarker research have opened new avenues for earlier detection and personalized oncology treatments, significantly impacting patient survival rates. The disease is increasingly prevalent globally, particularly in developed countries. Major risk factors include a diet rich in fats and processed red meats, physical inactivity, obesity, excessive alcohol consumption, and smoking. In addition to these, genetic predisposition plays a crucial role, with inherited mutations in genes such as APC, MLH1, and MSH2 being associated with cancer syndromes like familial adenomatous polyposis and Lynch syndrome. Chronic inflammation of the colon, as seen in ulcerative colitis and Crohn's disease, is another important risk factor. Patients with chronic inflammatory bowel diseases have a significantly higher risk of developing colorectal cancer due to prolonged exposure to a pro-inflammatory microenvironment that promotes uncontrolled proliferation of intestinal epithelial cells.

Colorectal cancer develops through a series of genetic and epigenetic alterations that drive the malignant transformation of colonic epithelial cells. Genomic instability is a key factor in its pathogenesis, occurring in two main forms. The first is microsatellite instability, found in patients with defects in the DNA mismatch repair system. This cancer subtype has a better prognosis and may respond well to immunotherapies. The second is chromosomal instability, marked by major alterations in chromosome number, favoring mutations in genes such as KRAS, TP53, and APC and associated with more aggressive disease forms. Epigenetic changes—such as abnormal methylation of tumor suppressor gene promoters—also contribute to tumor progression. For instance, hypermethylation of the MLH1 gene promoter leads to defective DNA repair and the accumulation of somatic mutations.

Chronic inflammation plays a determining role in the initiation and progression of colorectal cancer. Inflammatory cytokines contribute to the formation of a tumor-promoting microenvironment. Among the most studied cytokines in colorectal cancer are interleukin-8 (IL-8), interleukin-17 (IL-17), and interleukin-33 (IL-33). IL-8 stimulates angiogenesis and the recruitment of immunosuppressive cells, facilitating tumor growth and metastasis. IL-17 is involved in chronic inflammatory responses, promoting cellular proliferation and tissue remodeling. IL-33 plays a dual role, potentially fostering both antitumor immune responses and tumor progression depending on the biological context.

The tumor microenvironment consists of various non-neoplastic cells, including cancer-associated fibroblasts, endothelial cells, and tumor-associated macrophages, which contribute to immune evasion and neoplastic progression. Recent studies suggest that the interaction between these components and tumor cells can influence treatment response and guide the development of novel therapeutic approaches.

Recent research has focused on identifying clinically relevant biomarkers for early detection and disease monitoring. MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression and play essential roles in cancer progression. Studies have shown that miR-101-3p, miR-106a-5p, and miR-326 are dysregulated in colorectal cancer, with implications for cellular proliferation and treatment response. Inflammatory biomarkers such as IL-8, IL-17, and IL-33 have also been associated with colorectal cancer progression and poor prognosis. Epigenetic markers like DNA methylation of genes such as SEPT9 and SFRP2 show promise for early detection.

Advanced diagnostic techniques have been developed to enable early detection of colorectal cancer. Circulating tumor DNA tests can identify specific mutations in patient blood samples and are useful for monitoring treatment response. Techniques such as qRT-PCR and RNA sequencing allow precise quantification of genetic biomarkers, including miRNAs and interleukins. Molecular imaging techniques, including fluorescence-enhanced colonoscopy and functional MRI, provide detailed information on tumor metabolism and vascularization, facilitating more accurate diagnosis.

Colorectal cancer is a complex disease with multifactorial etiology and diverse pathogenic mechanisms. Advances in understanding the role of molecular and inflammatory biomarkers offer significant opportunities for developing early diagnostic methods and optimizing therapeutic strategies. The clinical use of interleukins and miRNAs may represent a key direction in precision medicine, enabling the identification of high-risk patients and personalized treatment based on individual tumor characteristics. Future studies should explore the integration of these biomarkers into clinical guidelines to improve prognosis for colorectal cancer patients.

General Methodology

The methodology employed in this doctoral thesis was designed to enable a comprehensive investigation of the immunological and genetic biomarkers involved in the pathogenesis of colorectal cancer. The study was conducted on a cohort of patients diagnosed with colorectal cancer, categorized according to disease stage, and a control group comprising healthy individuals. The primary objective of the research was to analyze the expression of interleukins and microRNAs in order to evaluate their potential as diagnostic and prognostic biomarkers.

Biological samples were collected from both patients and healthy controls via venous puncture, using vacutainer tubes containing anticoagulant. Immediately after

collection, the samples were centrifuged at 3000 rpm for 10 minutes to separate the plasma, which was then aliquoted and stored at −80°C until analysis. The cohort used for interleukin analysis included 42 colorectal cancer patients and 40 healthy controls. Blood samples were collected prior to the initiation of oncological treatment to eliminate any therapeutic influence on serum interleukin levels. In addition to plasma samples, tumoral and peritumoral tissue specimens were collected during surgery. Tissue fragments were harvested by specialized surgeons, immediately preserved in liquid nitrogen, and stored at −80°C for subsequent analysis. To avoid RNA degradation, the extraction process was carried out as rapidly as possible following collection. These tissue samples were used for the analysis of specific microRNAs involved in colorectal carcinogenesis. The microRNA cohort included 40 colorectal cancer patients from whom both tumor and peritumoral tissues were collected. This selection enabled a comparative analysis of microRNA expression between tumoral and peritumoral tissues, the latter serving as the internal control.

The serum levels of IL-8, IL-17, and IL-33 were quantified using the Enzyme-Linked Immunosorbent Assay (ELISA), a high-sensitivity technique that allows for the accurate measurement of cytokines in biological samples. The ELISA kits were chosen based on specificity and sensitivity, and absorbance readings were performed at 450 nm using a microplate spectrophotometer. Each analysis was conducted in duplicate to ensure the reproducibility of results, and the obtained values were compared between colorectal cancer patients and healthy controls.

For the analysis of miR-101-3p, miR-106a-5p, and miR-326 expression, total RNA was extracted from tumor and peritumoral tissues using specialized commercial kits. RNA extraction followed the manufacturer's protocol, which included cell lysis, purification, and removal of contaminants. RNA concentration and purity were assessed via spectrophotometry, and RNA integrity was verified through agarose gel electrophoresis. Subsequently, RNA was reverse-transcribed into complementary DNA (cDNA) using a specific kit, and amplification was performed by real-time polymerase chain reaction (qRT-PCR) using target-specific markers. U6 and GAPDH were used as reference genes, and data analysis was performed using the ΔΔCt method, allowing for the comparison of relative expression levels between study groups.

Statistical analysis was carried out using SPSS and GraphPad Prism software, applying both descriptive and inferential methods. Group comparisons were performed using Student's t-test and ANOVA, depending on data distribution. Correlations between interleukin levels, microRNA expression, and clinical parameters were assessed using Pearson and Spearman correlation coefficients. A statistical significance threshold was set at $p < 0.05$. Results were graphically represented using box plots and scatter plots to facilitate clear visual interpretation.

This methodological framework was chosen to ensure the accuracy and reproducibility of the data, allowing for an in-depth analysis of immunological and genetic biomarkers in colorectal cancer.

II. Personal contribution

Study I. miRNA Expression in the Staging of Colorectal Cancer

This study investigated the expression of three microRNAs—miR-101-3p, miR-106a-5p, and miR-326—in tumoral and peritumoral tissues from patients diagnosed with colorectal cancer, analyzing their correlation with tumor staging. The patient cohort included 40 individuals, distributed across the four TNM stages. The methods employed included total RNA extraction and expression analysis using qRT-PCR. This approach allowed for precise quantification of microRNA expression levels, revealing notable differences between tumoral and peritumoral tissues.

The expression levels of all three microRNAs were significantly higher in peritumoral tissues compared to tumoral ones, suggesting a possible tumor-suppressive role. In advanced stages of the disease, their expression was markedly reduced, particularly for miR-101-3p and miR-326, indicating an inverse correlation between disease progression and microRNA levels. miR-106a-5p showed relatively stable expression across most stages, with a noticeable peak in stage III, which may point to a distinct role in tumor progression.

Comparative analysis by gender and age group showed that miR-101-3p and miR-106a-5p were more highly expressed in younger female patients, while in older age groups, their levels were higher in males. miR-326 was significantly more expressed in females across most age groups, suggesting a potential hormonal influence on its expression. These observations indicate that miRNA expression is influenced not only by the presence of cancer but also by age and sex, which may have implications for understanding tumor biology and developing personalized diagnostic and therapeutic approaches.

The correlations among the three microRNAs were significant, suggesting interdependent expression in colorectal cancer. miR-101-3p was strongly and positively correlated with both miR-106a-5p and miR-326, suggesting a shared regulatory mechanism. Moreover, a strong correlation between miR-106a-5p and miR-326 was observed in advanced disease stages, highlighting their potential involvement in tumor progression. These relationships suggest that the expression levels of these miRNAs are part of a complex biological network influencing tumor growth and development.

The findings indicate that the reduced expression of miR-101-3p and miR-326 in tumoral tissues may have important functional implications, possibly playing roles in maintaining cellular homeostasis and suppressing malignant proliferation. Comparison

of miR-106a-5p expression between healthy and tumoral tissues suggested a moderate predictive relationship, while miR-326 showed no clear correlation between the two tissue types, indicating that their regulation may depend on multiple biological and environmental factors.

Functional analysis of these miRNAs revealed their involvement in the PI3K-Akt and MAPK signaling pathways, known for their roles in cancer progression. This supports the hypothesis that these microRNAs may significantly impact tumorigenesis regulation and represent potential therapeutic targets. Identifying such molecular links provides insight into how these miRNAs may influence cellular survival, proliferation, and differentiation in colorectal cancer.

This study demonstrated that miR-101-3p, miR-106a-5p, and miR-326 exhibit higher expression in peritumoral than in tumoral tissues and that their levels decrease in advanced stages of colorectal cancer. Significant correlations between these biomarkers suggest potential functional interdependence in carcinogenesis. These findings underscore the diagnostic and prognostic potential of these miRNAs in colorectal cancer and suggest their utility in disease screening and monitoring. The low expression of these miRNAs in advanced stages indicates a possible protective role, where their loss may facilitate tumor progression. Future studies on larger cohorts are necessary to validate their utility as biomarkers for early detection and disease monitoring, potentially enabling the development of personalized therapies based on the molecular profile of each patient. Integrating miRNA expression profiles with clinical parameters may offer a more detailed understanding of cancer biology and support the development of more effective disease management strategies.

Study II. miRNA Expression According to Tumor Grade, Invasion, and Localization in Colorectal Cancer

This study examined the expression patterns of miR-101-3p, miR-106a-5p, and miR-326 in colorectal cancer, evaluating their correlations with tumor grade, tissue invasion depth, and anatomical localization. The results revealed significant variations in microRNA expression depending on these parameters, suggesting that these molecules may serve as diagnostic and prognostic biomarkers.

With respect to tumor differentiation grade, miR-101-3p and miR-326 were more highly expressed in moderately differentiated (G2) tumors but decreased in poorly differentiated (G3) tumors. This pattern suggests a potential role in the transition toward a more aggressive tumor phenotype. miR-106a-5p followed a distinct trajectory, displaying the highest expression in G2 tumors and a significant reduction in G3, pointing to a key role in the intermediate stages of tumor progression. These expression dynamics imply a potential involvement of these miRNAs in tumor cell proliferation and differentiation mechanisms.

Invasion analysis revealed significant differences in microRNA expression across various invasion types. miR-101-3p showed elevated expression in tumors with muscular invasion, possibly indicating a role in the early stages of tumor expansion. miR-106a-5p reached its highest levels in tumors with lymphovascular invasion, suggesting a role in the dissemination of tumor cells via the circulatory system. miR-326 was most highly expressed in tumors with distant organ metastases, indicating a strong association with highly aggressive tumor phenotypes. These findings highlight a link between miRNA expression profiles and the severity of tumor invasion.

MicroRNA expression also varied significantly depending on tumor localization. Levels of miR-101-3p, miR-106a-5p, and miR-326 were higher in tumors located in the left colon compared to those in the right colon. This difference was most pronounced for miR-106a-5p, suggesting distinct oncogenic mechanisms between tumors originating from these two anatomical regions. The lower expression observed in right-sided tumors may reflect molecular features such as microsatellite instability and variations in signaling pathways.

Correlations between microRNAs demonstrated significant interactions among these molecular markers. miR-101-3p and miR-106a-5p exhibited a positive relationship, with increasing expression in higher-grade tumors, particularly in G3. miR-101-3p and miR-326 were also correlated, suggesting potential co-regulation during early stages of tumor progression. miR-106a-5p and miR-326 were strongly associated in tumors with distant metastases and lymphovascular invasion, indicating a shared contribution to tumor aggressiveness. Multiple regression analyses confirmed these relationships, suggesting that these miRNAs are interdependent and may serve as predictive markers of colorectal cancer progression.

These findings underscore the relevance of microRNAs as biomarkers for colorectal cancer characterization, providing valuable insights into the molecular mechanisms of disease progression. The study emphasizes the potential of using these miRNAs in early diagnosis, patient stratification, and the development of targeted therapies, based on their specific expression patterns in relation to tumor grade, invasion, and localization.

Study III. Interleukins 8, 17A, and 33 as Potential Biomarkers in Colorectal Cancer

Interleukins IL-8, IL-17A, and IL-33 play critical roles in colorectal cancer progression by influencing inflammation, angiogenesis, and the tumor microenvironment. The expression of these interleukins varies according to disease stage, patient age, and sex, with important implications for diagnosis and prognosis. This study included 42 patients diagnosed with colorectal cancer and 40 healthy controls. Serum levels of IL-8, IL-17A, and IL-33 were measured using the ELISA technique.

IL-8 expression was significantly higher in colorectal cancer patients compared to healthy subjects, with a mean value of 29.15 pg/mL versus 19.16 pg/mL. The highest concentrations were observed in the early stages of the disease, particularly stage I (34.6 pg/mL) and stage II (32.29 pg/mL), followed by a progressive decline in advanced stages. These correlations indicate that IL-8 is a strong inflammatory marker with a major role in tumor initiation, significantly influencing angiogenesis and cell migration.

IL-17A expression was also elevated in colorectal cancer patients, with a mean of 26.05 pg/mL compared to 7.10 pg/mL in healthy controls. The highest expression was recorded in the 60–69 age group, peaking at 43.5 pg/mL, followed by a marked decline in older patients. By disease stage, IL-17A levels were highest in stage I (32.1 pg/mL) and stage II (35.77 pg/mL), with a dramatic drop in stage IV (7.29 pg/mL). This decrease in advanced disease may reflect the negative regulation of the inflammatory response as tumors progress, potentially due to immunosuppressive mechanisms within the tumor microenvironment.

IL-33 showed an inverse pattern, with significantly higher levels in healthy individuals (121.4 pg/mL) than in cancer patients (42.47 pg/mL), suggesting a possible protective role in maintaining tissue homeostasis. By cancer stage, IL-33 levels were highest in stage I (48.4 pg/mL) and stage II (46.5 pg/mL), gradually declining in more advanced stages. This dynamic suggests that IL-33 may act initially as a pro-inflammatory mediator, but is later suppressed as the tumor develops immune evasion mechanisms.

Correlations between interleukins revealed significant relationships, particularly between IL-8 and IL-17A, which showed a positive association in cancer patients ($p = 0.001$), indicating an interdependence between systemic inflammation and tumor progression. This correlation was absent in healthy individuals, suggesting that the interaction between these interleukins is specific to the tumor microenvironment. The relationship between IL-33 and IL-17A was also significant ($p = 0.007$), but exhibited a nonlinear pattern, suggesting a complex regulatory mechanism in colorectal cancer. No significant correlation was found between IL-8 and IL-33, indicating that these cytokines likely act through distinct pathways within the disease context.

Sex-based differences in expression showed higher interleukin levels in males than females, particularly for IL-8 (38.94 pg/mL vs. 21.07 pg/mL) and IL-17A (33.31 pg/mL vs. 20.06 pg/mL). These results suggest a stronger inflammatory response in males, potentially associated with more aggressive disease progression. With respect to age, interleukin expression peaked in the 60–69 age group, indicating a critical window for the development and progression of colorectal cancer.

This study supports the concept that IL-8, IL-17A, and IL-33 are involved in both inflammatory processes and tumor progression, highlighting their potential as

biomarkers for colorectal cancer staging. The elevated levels of IL-8 and IL-17A in early stages suggest their utility for early diagnosis and monitoring treatment response. Conversely, the decreased IL-33 expression in cancer patients raises questions about its exact role, warranting further investigation into its function in tumorigenesis. The results confirm the importance of profiling inflammatory markers in colorectal cancer and underscore the need for additional research to better understand interleukin interactions. Future studies should also investigate the expression of microRNAs regulating these interleukins to elucidate transcriptional and post-transcriptional mechanisms involved in colorectal cancer progression.

Study IV. The Impact of Tumor Localization, Grade, and Invasion on Interleukin Expression in Colorectal Cancer

This study examined the expression of interleukins IL-8, IL-17A, and IL-33 in colorectal cancer, taking into account tumor localization, histological grade, and depth of invasion. These interleukins play critical roles in tumor progression by modulating inflammation, immune response, and angiogenesis. Their expression varied depending on the analyzed parameters, with potential implications for prognosis and the development of targeted therapies.

IL-8 expression was found to be higher in tumors located in the left colon compared to those in the right colon, suggesting a more intense inflammatory response in the left-sided colorectal segment. IL-17A and IL-33 also exhibited slightly higher levels in left-sided tumors, possibly reflecting a stronger immune response or distinct interactions with the tumor microenvironment. These expression differences may be attributed to anatomical and physiological variations between the two segments of the colon and to divergent tumorigenic mechanisms.

Regarding histological grade, IL-8 expression was highest in well-differentiated tumors (G1), but showed wide variability in moderately differentiated tumors (G2). This observation supports the hypothesis of IL-8's strong pro-inflammatory role during early tumor development, followed by a decrease as tumors become more aggressive. IL-17A and IL-33 followed a similar pattern, with higher expression in well-differentiated tumors and progressive reduction in poorly differentiated tumors (G3). These results may suggest an initially protective function of IL-17A and IL-33, which is subsequently downregulated as the immune response becomes suppressed in advanced disease.

With respect to invasion depth, IL-8 levels were elevated in tumors invading the serosa and submucosal layers, indicating its involvement in promoting metastatic potential. IL-17A peaked in tumors with submucosal invasion but dropped significantly in those with distant organ metastases, reflecting reduced immune response in advanced stages. IL-33 showed increased levels in tumors invading the muscular and serosal

layers, suggesting its involvement in deep tissue invasion and remodeling of the tumor microenvironment.

Analysis of correlations between interleukins revealed differential relationships depending on tumor grade and invasion. A significant but nonlinear correlation was observed between IL-8 and IL-17A, with IL-17A initially increasing alongside IL-8 but decreasing beyond a certain threshold, particularly in poorly differentiated tumors. This suggests a complex regulatory mechanism of inflammation in colorectal cancer. No significant correlation was found between IL-8 and IL-33, indicating that these cytokines may act independently in tumor progression. Similarly, IL-33 did not appear to influence IL-17A directly, suggesting they are regulated by distinct factors within the tumor microenvironment.

Interleukin expression also varied according to patient age and sex. Patients over 70 more frequently had well-differentiated tumors located in the right colon, while older women with poorly differentiated tumors were more prevalent than their male counterparts. These findings may be relevant for understanding disease progression and for adapting therapeutic strategies accordingly.

In conclusion, IL-8, IL-17A, and IL-33 are actively involved in colorectal cancer progression, with expression patterns influenced by tumor location, histological grade, and invasion depth. The elevated levels of IL-8 and IL-17A in early disease stages suggest a strong pro-inflammatory role, while the reduction of IL-17A and IL-33 in poorly differentiated tumors may indicate immune suppression in later stages. IL-8 appears to be a key factor in tumor invasion and metastasis, while IL-33 plays a more ambivalent role, with both pro- and anti-tumor potential. These findings support the use of interleukins as potential biomarkers for the diagnosis and treatment of colorectal cancer.

Originality and Contributions of the Thesis

This doctoral thesis offers essential contributions to the fields of molecular and immunological oncology, providing innovative perspectives on the mechanisms involved in colorectal cancer. The originality of the work lies in its comprehensive approach to the study of genetic and immunological biomarkers and in its proposal of new research directions for early diagnosis and personalized therapy.

One of the main contributions of the thesis is the identification of relevant genetic and immunological biomarkers for the staging of colorectal cancer. The study demonstrates the potential role of microRNAs miR-101-3p, miR-106a-5p, and miR-326 as biomarkers, by analyzing their expression in both tumoral and peritumoral tissues. The correlation between the expression levels of these microRNAs and cancer progression suggests that their downregulation in advanced stages reflects a critical role in regulating oncogenesis and related molecular pathways.

Another major contribution is the implementation of the first study to investigate the circulating correlations between interleukins IL-8 and IL-17A in colorectal cancer. This research reveals a significant interdependence between these interleukins, contributing to a deeper understanding of the inflammatory processes surrounding malignant tumors and highlighting new potential therapeutic targets. Additionally, the thesis shows the potential of IL-33 as an anti-inflammatory biomarker, with elevated levels found in the control group compared to colorectal cancer patients. This finding suggests a possible protective role for IL-33 in the early stages of the disease and opens new avenues of research into the influence of immune responses on tumor progression.

The originality of the study is further reinforced by the use of multiple regression analysis to explore interleukin interdependencies. This statistical approach confirms the relationship between IL-8, IL-17A, and IL-33 based on tumor grade and highlights the interaction mechanisms that may influence the course of colorectal cancer. These correlations represent a significant contribution to the development of new biomarker-based strategies for diagnosis and treatment.

Through this research, the thesis offers an in-depth understanding of the molecular and immunological processes involved in colorectal cancer, supporting the development of early diagnostic methods and more effective targeted therapies. The work represents a substantial advancement in the field of personalized oncology, with a meaningful impact on the future of therapeutic approaches in this pathology.