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Identification of microRNAs Expression Pattern in Colorectal Cancer Patients and its Favorable Modulation after Gut Microbiota Enrichment with Probiotics

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Abstract

Colorectal cancer (CRC) represents the third most commonly diagnosed tumor and the second cause of cancer-related death worldwide. Despite diagnostic and therapeutic advances, the mortality rates of CRC remain high, especially in industrialized countries. Numerous studies have demonstrated how several factors are involved in the development and progression of CRC. Among these, epigenetic modifications and intestinal microbiota seems to play a pivotal role in the pathogenesis of CRC, however, the precise mechanisms driven by epigenetic factors, like microRNAs (miRNAs), and gut microbiota have not been fully elucidated yet. Notably, gut microbiota is considered a real neglected organ. In the last decade, several pre-clinical and clinical studies performed by using novel high-throughput technologies (i.e., metagenomics, metatranscriptomics, metabolomics), have allowed the identification of a crosstalk between gut microbiota and cancer, especially in patients with CRC, demonstrating how specific bacteria may increase the risk of development of tumors. In this context, it was also demonstrated that eubiotic microbiota exerts protective mechanisms against the detrimental effects of carcinogens as well as reduce the side effects of pharmacological therapies (e.g., diarrhea, mucositis, etc.) in cancer patients.

As regards miRNAs, several studies have tried to identify miRNAs specifically associated with the presence of CRC or with the prognosis of this tumor, however, very confusing data have been generated and no reliable biomarkers are currently recognized effective in diagnosing this tumor.

Therefore, although a great interest is currently devoted on both miRNAs and gut microbiota as potential indicators and predictors of CRC development, there is a lack of effective biomarkers for the early diagnosis of this tumor which suggest the need of further investigations about these two promising topics.

On these bases, the aim of the present study was to further establish the potential diagnostic and prognostic role of miRNAs in CRC as well as the impact of gut microbiota and its modulation in the expression levels of miRNAs known to be involved in the development and progression of this tumor.

For these purposes, first, computational analyses were performed by analyzing miRNA expression profile of CRC patients by using the data contained in the Gene Expression Omnibus DataSets (GEO DataSets) and The Cancer Genome Atlas databases (TCGA). Unlike other similar bioinformatics studies, in this study an integrated computational analysis was performed by considering all the relevant microarray miRNA expression datasets on CRC patients and the miRNA-seq data of GEO DataSets and TCGA databses.

Through these analyses, it was possible to identify a list of miRNAs associated with the development of CRC. Further bioinformatics analyses performed by using different prediction tools allow us to establish the functional role of the selected miRNAs in CRC. Among the miRNAs identified, four different miRNAs, i.e. hsa-miR-21-5p, hsa-miR-503-5p, hsa-miR-497-5p and hsa-miR-375, were selected for the validation analyses on CRC tissue and liquid biopsy samples and as miRNAs potentially modulated by the modification of gut microbiota obtained through probiotic enrichment.

From an experimental point of view, the dysregulation of the expression levels of the four selected miRNAs were evaluated in both liquid biopsy samples and Formalin-Fixed Paraffin Embedded (FFPE) tissue samples and adjacent normal mucosa obtained from CRC patients. For these validation analyses, the high-sensitive droplet digital PCR (ddPCR) amplification systems was used.

The ddPCR analyses performed on CRC FFPE samples and normal mucosa confirmed the preliminary computational data showing the strong up-regulation of the two predicted overexpressed miRNAs, hsa-miR-21-5p and hsa-miR-503-5p, and the significant down-regulation of the two predicted down-regulated hsa-miR-497-5p and hsa-miR-375.

As regards the analysis of liquid biopsy samples, only two miRNAs were significantly dysregulated in CRC patients compared to healthy donors. Indeed, according to the computational results previously obtained, circulating hsa-miR-21-5p resulted up-regulated while the hsa-miR-497-5p was down-regulated in CRC patients. The high diagnostic significance of the selected miRNAs was further confirmed by performing Receiver Operating Characteristic (ROC) analyses.

Regarding the modulating effects induced by probiotics and microbiota enrichment in the expression levels of miRNAs another GEO DataSets platform containing miRNA expression levels in samples before and after probiotic intervention was analyzed. By merging the list of miRNAs involved in CRC with those modulated by probiotics intervention, we confirmed the involvement of the previous analyzed miRNAs in CRC.

Finally, to validate the modulating potential of probiotics intervention, the expression levels of the three selected miRNAs were investigated on liquid biopsy samples of CRC patients before and after three months of *Lactobacillus rhamnosus* GG (LGG) probiotic administration selected within the participants of a clinical trail on the “Maintenance of normal gastro-intestinal function with dietary supplement containing *Lactobacillus rhamnosus* GG in cancer patients treated with cytotoxic chemotherapy and/or targeted therapy”.

These further analyses confirmed the modulatory effects of LGG on the expression levels of miRNAs involved with the development and progression of CRC.

Overall, this study represents the starting point for the adoption of miRNAs and probiotics for the management of CRC. More in detail, after miRNA validation in a wider cohort of CRC patients, the miRNAs hsa-miR-21-5p, hsa-miR-503-5p, hsa-miR-497-5p and hsa-miR-375 could be proposed as novel and high-sensitive biomarkers for the diagnosis of CRC. In addition, the data here obtained confirm the direct and indirect beneficial effects of probiotics administration in CRC patients by reducing the gastrointestinal toxicity of chemotherapy and inducing positive modulations of miRNAs involved in CRC progression. Finally, the data here obtained strongly encourage the use of ddPCR as an effective and sensitive analytical method to evaluate the expression levels of miRNAs both in tissues and liquid biopsy samples. This high-sensitive strategy could be applied for the development of novel effective screening programs and for the construction of novel diagnostic card or platform useful for the non-invasive and early diagnosis of CRC in individuals at risk for this tumor.

TABLE OF CONTENTS

1. INTRODUCTION 1

1.1 Colorectal Cancer 1

1.1.1 Microscopical characteristics 1

1.1.2 TNM Staging and tumor grading 1

1.1.3 Epidemiological data 2

1.1.4 Risk factors and pathogenetic mechanisms 3

1.1.5 Molecular determinants of CRC: genetic and epigenetic alterations . 8

1.1.6 Early diagnosis of CRC: Screening and diagnostic strategies 9

1.1.7 CRC Treatment: Surgical resection and pharmacological strategies 12

1.2 microRNAs 15

1.2.1 Biogenesis and mechanism of action 15

1.2.2 Dysregulated miRNAs in CRC 17

1.3 Gut microbiota 18

1.3.1 Gut microbiota and cancer 18

1.3.2 Involvement of Gut microbiota in neoplastic transformation mechanism 24

1.3.3. Involvement of Gut Microbiota in Colorectal Cancer 29

2. AIM OF THE STUDY 34

3. MATERIALS AND METHODS 35

3.1 Computational identification of microRNAs involved in the development and progression of colorectal cancer and modulated by probiotics interventions 35

3.2 Association between the most dysregulated miRNAs and the Main Genes Altered in Colorectal Cancer 37

3.3 Functional role of selected miRNAs and modulated molecular pathways 38

3.4 Patients and sample included in the study 38

3.5 RNA Extraction and microRNA Reverse Transcription 39

3.6 Absolute quantification of miRNAs expression levels 40

3.7 Bioinformatics analyses **41**

3.8 Statistical analyses **42**

4. RESULTS **44**

4.1 Computational identification of miRNAs associated with colorectal cancer development **44**

4.2 Clinical validation of the diagnostic role of the four selected miRNAs in Colorectal Cancer samples **61**

4.3 Bioinformatics evaluation of the prognostic value of the four selected miRNAs **67**

4.4 Positive miRNA modulation induced by microbial interventions with the administration of probiotics in CRC patients **75**

5. DISCUSSION **80**

6. CONCLUSIONS **86**

7. REFERENCES **88**

Appendix A **110**

1. INTRODUCTION

1.1 Colorectal Cancer

1.1.1 Microscopical characteristics

About 85% of Colorectal Cancer (CRC) are represented by adenocarcinomas. By evaluating both histological and microscopic characteristics it is possible to distinguish different types of CRC [Hedinger C et al., 1989]:

- Mucinous adenocarcinoma: consists of neoplastic cells that have a high mucin content [Luo C. et al., 2019];
- Signet-Ring Cell Carcinoma (SRCC): characterized by the presence of a large vacuole of mucin inside the cell that confines the nucleus in a peripheral position [An Y. et al., 2021];
- Undifferentiated carcinoma: does not show any sign of typical epithelial differentiation [Ashitomi Y. et al., 2020];
- Squamous carcinoma: neoplastic cells show a squamous morphology [Jahromi G N. 2020];
- Small-cell carcinoma: a very rare disease among colon neoplasms [Iwase T. et al., 2013];
- Adenosquamous carcinoma: neoplastic cells are characterized by mixed morphology between squamous and glandular types [Sunkara T. et al., 2018].

1.1.2 TNM Staging and tumor grading

In order to perform an adequate therapeutic approach and have prognostic indications on the neoplasm it is necessary to determine the stage of the tumor. It is important to differentiate between tumor staging and tumor grading. Indeed, the first takes into account the size of tumor, the involvement of lymph nodes and the presence of metastases, while the latter indicates the differentiation grade of the neoplastic cell. In particular, well-differentiated grade 1 cells have morphological and structural characteristics similar to normal cells; vice versa,

the most undifferentiated cells lose these characteristics. Currently the staging classification system for CRC is represented by the TNM System of the American Joint Committee on Cancer (AJCC). TNM is an acronym in which each letter stands for a different tumor feature: T stands for tumor extension, N indicates Lymph node involvement and M stands for presence of metastasis. In particular, on the basis of these three parameters, the TNM system classifies the CRC into 4 stages which, with the exception of stages 0 and 1, can be divided into further subgroups (0, I, IIA, IIB, IIC, IIIA, IIIB, IIIC, IVA, IVB, IVC) [AIOM 2021].

1.1.3 Epidemiological data

CRC is one of the most frequently diagnosed cancers in the world. It represents the third most common type of cancer with the highest incidence in the world (1,931,590 cases - 10%) behind only lung (11.4%) and breast cancer (11.7%). Age-standardized epidemiological data show how this tumor is more frequently diagnosed in males, which shows incidence rates of 23.4/100,000 compared to 16.2/100,000 of females. The incidence of this tumor appears to be related to geographic distribution. In particular, Asia and Europe are the geographical areas with the highest incidence rate for CRC with 1,009,400 (52.3%) and 519,820 (26.9%) new diagnoses, respectively; Northern America, Latin America and the Caribbean, Africa and Oceania, on the other hand, have lower incidence rates (GLOBOCAN 2020).

Despite the advancement of therapeutic and diagnostic strategies, colorectal cancer still represents one of the tumors with the highest number of annual deaths. According to GLOBOCAN data, in 2020, 935,173 (9.4%) deaths from CRC were recorded; these data indicated that CRC is the second cancer with the highest mortality rate in the world behind only lung cancer with 1,796,144 (18%) annually deaths. According to geographic distribution, mortality rates follow a similar trend to incidence rates. Indeed, the continents with the highest mortality rates for CRC are Asia and Europe with 506,449 (54.2%) and 244,824 (26.2%) deaths, respectively, followed by Latin America and the Caribbean, Northern America, Africa and Oceania with gradually decreasing rates.

According to the epidemiological data reported in 2021 by the collaboration of the AIOM-AIRTUM research groups, CRC is one of the three cancers with the highest incidence rate in Italy. Indeed, in 2020 approximately 43,700 new diagnoses for CRC were estimated, with higher incidence rates in men (23,400 cases) compared to women (20,300 cases). According to these data, in 2021 the estimated death rate for males was 12.1/100,000 with a decrease of 13.6% compared to 2015. A similar trend was also observed for females with a decrease of 13.2% compared to 2015. This is due to the efficient screening programs that have involved about 2 millions of people aged between 50 and 74 years, allowing to identify the presence of CRC in early stage and treat them with appropriate therapeutic strategies. Furthermore, the 5-years survivals rates for CRC have an average value of 65% and 66% for male and female, respectively. These rates decrease significantly in older patients than in younger patients, reaching values of 51.8% in men over 75 years of age (AIOM-AIRTUM 2021).

1.1.4 Risk factors and pathogenetic mechanisms

Different studies have identified different risk factors associated an increased risk of development of colorectal cancer; these can be grouped into two main categories. In particular, the onset of CRC may be due to the exposure to modifiable risk factors or non-modifiable risk factors. The two categories are not mutually exclusive; indeed some individuals may be exposed to both types of risk factors.

1.1.4.1 Modifiable risk factors

This category includes all those environmental factors that can be modified or even eliminated by modifying the individual habits. In particular, nutritional behavior, cigarette smoking and alcohol consumption are part of this class.
Regarding the dietary habits, low consumption of fiber and vegetables and low intake of foods rich in vitamins and antioxidants nutrients are among the most harmful eating habits. The decrease or total absence of fiber in diet leads to an increased risk of developing CRC due to a double synergistic effect. Indeed, the main consequences are the alteration of the intestinal microbiota, a reduction in fecal mass and an increase in the harmful effects caused by waste by-products

derived by microbial metabolism. Normally these foods are also rich in vitamins, therefore, their low consumption also determines the lack of the protective and antioxidant effects of various vitamins such as vitamins A, C and E [Kumar V. et al.,2012]. Other harmful eating habits are excessive consumption of processed meats and foods with a high fat content. Fat foods increase the content of fatty acids which lead to an increase in the synthesis of cholesterol and bile acids. High levels of these substances are potentially harmful because they can be converted into carcinogenic products by intestinal microbiota. Different studies have also shown that excessive consumption of processed meat (50g per day) leads to an increase of 18% in the risk of developing distal-colon cancer. Furthermore, excessive consumption of red meat can also increase the risk of developing this cancer; in this case, the risk associated with red meat consumption of 100g per day is 17% [Chao A. et al., 2005; Chan DS et al., 2011]. Another evidence of the potential harmful effect of these foods is provided by the World Health Organization's International Agency for Research on Cancer which in 2015 classified processed meat as class 1 carcinogen, and red meat in class 2A [Bouvard V. et al., 2015].
In the category of modifiable risk factors there are also cigarette smoking and alcohol consumption. As demonstrated for other cancers, cigarette smoking has been associated with an increased risk of developing colon cancer. In particular, Amitay and colleagues showed that smokers enrolled in their study had a 59% increased risk of developing CRC compared to non-smokers [Amitay EL. Et al., 2020]. An increased risk of developing this cancer is also associated with excessive alcohol consumption; it has been reported that individuals who drink 2-3 alcoholic beverages per day have a 20% increased risk of developing colon cancer. This risk increases to 40% if consumption exceeds three drinks per day [Rawla P et al., 2019].

1.1.4.2 Unmodifiable risk factors: Genetic and epigenetic alterations in colorectal cancer

Non-modifiable risk factors are those factors that cannot be changed by changing an individual's behavioral habits. Most cases of CRC are associated with genetic alterations due to somatic mutations while only a small percentage of them are

caused by hereditary syndromes. In particular, Lynch syndrome and familial adenomatous polyposis (FAP) are responsible for about 5% of CRC cases [Stoffel EM et al., 2014]. Lynch syndrome, also called hereditary nonpolyposis colorectal cancer (HNPCC), increases the risk of developing a CRC by 80%. It is caused by the presence of germline autosomal dominant mutations with 90% penetrance affecting genes involved in the correction of DNA mutations occurring during DNA replication. These are represented by MLH1, PMS1, PMS2, MSH2 and MSH6 which are involved in the mismatch repair (MMR) mechanisms [Nagy R. et al., 2004]. When these genes are mutated, their main functions are compromised; this causes a failure of genetic replication and recombination errors during the DNA replication resulting in the formation of mismatched nucleotides. The lack of this correction system leads to an accumulation of mutations mainly in highly repeated DNA sequences called microsatellites and can lead to the alteration of key processes in the mechanisms of carcinogenesis such as cell growth and apoptosis; this condition is called microsatellite instability (MSI) [Charames GS et al., 2003].

These alterations can also be found in non-hereditary colorectal cancers. In particular, in these cases there is an alteration of the MMR system due to somatic mutations; an example is represented by a down-regulation of gene expression due to promoter methylation. The diagnosis of Lynch syndrome is based on the search for germline mutations in the MMR genes. To identify the patients who must undergo the analysis, several criteria must be applied:

- Familiar history: an early evaluation with Bethesda Guidelines and clinical evaluation such as Amsterdam criteria;
- Tumor-based test: MSI testing and Immunohistochemistry (IHC) analysis [Giardiello FM. Et al., 2014];
- Foresight models: MMRpredict, MMRpro, PREMM [Vasen HF et al., 1991; Vasen HF et al., 1999; Umar A. et al., 2004].

Another hereditary syndrome involved in the onset of CRC is Familial Adenomatous Polyposis (FAP). It is responsible for about 1% of CRC [Wennstrom J. et al., 1974]. Unlike HNPCC, FAP results in the formation of many polyps/adenomas in the colon and rectum. This is an autosomal dominant

syndrome with complete penetrance that is caused by the mutation of the tumor suppressor gene APC (5q21-q22). Other evidence suggests that a second sporadic mutation in the non-mutated allele of the gene (two-hit hypothesis) causes an increase in the speed of onset of adenomas and their neoplastic transformation in association with further gene mutations such as those affecting the p53 gene [Zhang L et al., 2017]. The APC gene is responsible for the transcription of the homonymous protein called Adenomatous Polyposis Coli (APC) which plays a significant role as a tumor suppressor by interacting with different mechanisms including the Wnt/β-catenin pathway. The APC protein is able to regulate the cytoplasmic levels of β-catenin allowing its ubiquitin-dependent proteasomal degradation thanks to the action of the Axin-APC degradosome complex. When the gene is mutated, the APC protein loses its function; this determines an accumulation of β-catenin which by translocating in the nucleus will allow the transcription of cyclin D1 and MYC which are involved in growth, cell proliferation, cell-cycle progression and apoptosis [Cheng X et al., 2019; Ranes M et al., 2021].

The alteration of the Wnt/β-catenin pathway also interacts with the levels of Cyclooxygenase 2 (COX2) [Nunez F. et al., 2011]. COX2 levels appear to be increased in the presence of colonic adenomas and are also associated with adenomas predictive features of neoplastic transformation [McLean MH. et al., 2008]. Different studies have aimed to investigate the effects of non-steroidal anti-inflammatory drugs (NSAIDs) as chemopreventive agents in colorectal carcinogenesis. In particular, it has been highlighted that the administration of celecoxib, a selective COX-2 inhibitor, could lead to a decrease in the number of polyps in patients with FAP [Kemp Bohan PM. et al., 2021]. However, the use of these drugs, in particular of selective COX-2 inhibitors such as celocoxib, have been associated with an increased risk of cardiovascular events [Yang P. et al., 2022]. Furthermore, other studies have shown how the administration of Aspirin, a non-selective COX inhibitor that inhibits both COX-1 and COX-2, resulted in a decrease in the incidence and mortality associated to CRC [Rothwell PM. et al., 2010; Patrignani P. et al., 2016].

The APC gene has a mutation cluster region, where mutations accumulate over time; these can be represented by inactivating somatic mutations of APC or also by methylation of the promoter with consequent down-regulation of its expression. In this regard, the APC gene mutation was identified in 80% of sporadic CRC as well as in some cancer of the stomach, pancreas, thyroid, ovary [Brown SL et al., 2007].

About 30% of CRCs are referred to as familial-type. Although the terms hereditary and familial are often confused and misused, familial CRCs are those tumors that do not have specific inheritance patterns but are instead characterized by a higher than expected incidence within a family [Giglia MD et al., 2016]. In this regard, new studies have shown the existence of a new form of familial CRC called "Familial colorectal cancer type X" (FCCTX). This is a form of CRC that presents a cancer pattern similar to Lynch syndrome whose genetic basis seems to be associated with the presence of alterations of several genes such as BMPR1A, RPS20, SEMA4A, SETD6, OGG1 and FAN1 [Garcia FAO et al., 2022].

The largest number of diagnosed CRC is not linked to hereditary or familial alterations; indeed, these tumors could be caused by low penetrance somatic mutations and polymorphisms. These mutations can be induced by the presence of pathological conditions that can disturb normal homeostasis in the intestinal mucosa. Scientific evidence has suggested that inflammatory bowel disease (IBD) can increase the risk of developing CRC; in particular, it was highlighted that several patients with IBD had high levels of free radicals associated with alterations of genes involved in carcinogenesis processes such as p53 mutations, MSI and hypermethylation of the MLH1 gene [Stidham RW. et al., 2018].

Other studies have shown an involvement of diabetes in the onset of CRC. In particular, type 2 diabetes mellitus, characterized by increased production of insulin due to insulin resistance, appears to be epidemiologically linked to CRC [Ling S. et al., 2022]. Furthermore, it has been shown that high insulin levels could determine the onset of CRC through different pro-mitogenic, angiogenic, and anti-apoptotic effects directed on tumor cells or by activating the Wnt/β-

catenin signaling pathway through GSK-3β downregulation [Jain S. et al., 2017; Bertrand F.E. et al., 2020; Vekic J. Et al., 2021].

1.1.5 Molecular determinants of CRC: genetic and epigenetic alterations

Although MMR machinery and APC/β-catenin pathway are the most common altered pathways associated with CRC, different studies showed the presence of other dysregulated molecular mechanisms in CRC such as the MAPKinase pathway. In particular, in CRC, this pathway appears to be deregulated in the presence of EphB2 gene mutations (genomic loss or promoter methylation) or activating mutations in the KRAS and BRAF genes [Liu W. et al., 2022].

KRAS gene, located on chromosome 12 (12p12.1), is a protooncogene encoding for a GTPase involved in the signaling transduction of the MAPK pathway. Briefly, the stimulation of the epidermal growth factor receptor activates the tyrosine kinase activity of the cytoplasmic domain of the receptor which through a series of intermediate proteins (GRB2 and SOS) allows the activation of RAS by binding with GTP. Activated Ras then activates the protein kinase activity of a RAF kinase which activates other downstream proteins via a signaling cascade regulating different processes such as cell proliferation, differentiation, and apoptosis. In particular, it has been shown that activating mutations of KRAS or its target BRAF can occur in various cases of CRC, leading to an uncontrolled activation of the MAPK pathway. The hyperactivation of the MAPK pathway is associated with an increase of many processes involved in the carcinogenesis mechanism [Yamakuchi M et al., 2008; Lu H et al., 2010; Burotto M. et al., 2015; Zhu G. et al., 2021; Motta R. et al., 2021]. Furthermore, other studies have shown that alterations in these processes may also depend on mutations involving the PI3K pathway, and TGFB1- SMAD pathway [Yamakuchi M et al., 2008; Müller MF. et al., 2016; Proximal husbands A. et al., 2020].

Although genetic alterations play an important role in the onset of CRC, several studies suggest that CRC may also be associated with epigenetic alterations such as aberrant DNA methylation phenomena and the expression of non-coding RNA such as microRNAs (miRNAs) [Jung G. et al., 2020].

DNA methylation is a process finely regulated by several enzymes called DNA methyl transferases (DNMTs) (DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L) [Goll et al., 2005; Li et al., 2007], which determine the covalent addition of a methyl group from S-adenosyl-L-methionine (SAM), to the cytosine carbon in position 5', forming a 5-methyl-cytosine (5me). In humans, the addition of methyl groups mainly occurs in specific dinucleotides consisting of a cytosine preceding a guanine, called CpG dinucleotides [Rideout et al., 1990]; these are distributed in different areas of the genome containing highly repeated sequences such as centromeres or retrotransposomes, and small areas of DNA containing at least 200 bp called CpG Islands [Bird et al., 2002; Takai et al., 2002]. During tumor development, the methylation pattern of the normal cells undergoes various alterations; this epigenetic alteration was the first to be discovered in cancer [Feinberg et al., 1983; Riggs et al., 1983]. It seems that these modifications characterized by both extensive hypomethylation and hypermethylation phenomena [Jones et al., 2002; Gal-Yam et al., 2008] may stimulate tumor development and progression by altering gene expression [Feinberg et al., 2006]. Several studies have shown how aberrant methylation can be involved in the onset of CRC; in particular, a down-regulation of several tumor-suppressor genes such as CDKN2A, MLH1 and APC has been described due to promoter hypermethylation [Liang TJ. Et al., 2017; Jung G. et al., 2020]. In addition, other studies have shown a hyperactivation of various proto-oncogenes such as MYC and HRAS due to hypomethylation phenomena [Luo J. Et al., 2010; Jung G. et al., 2020].

1.1.6 Early diagnosis of CRC: Screening and diagnostic strategies

The screening programs represent a secondary prevention method that allow the early identification of tumors making possible timely and more effective therapeutic interventions with better outcomes for the patients' prognosis. In order to implement these programs for a specific type of cancer, the following guidelines were developed:

- Starting a screening program is very expensive. Therefore, this high cost must be offset by high tumor spread (high incidence and mortality rates);

- The time between the onset of the tumor and its pathological evolution should be long. This favor the early identification of tumor before its spreading;
- Screening tests with high sensitivity and specificity should be available for that specific tumor;
- Starting therapeutic treatments when the tumor is at an early stage should ensure an improvement in the patients prognosis;
- Availability of non-invasive and economic diagnostic screening methods with good compliance.

Screening methods are also available for CRC; these have made it possible to decrease the mortality rate associated with CRC in many countries. In particular, Edwards and colleagues showed that application of secondary prevention programs in the USA resulted in a 53% decrease in the mortality rate associated with this cancer [Edwards BK et al., 2010].

One of the screening methods available for CRC in Italy is represented by the test for the detection of fecal occult blood. Despite its speed of execution and its high tolerability by the patient, it is characterized by a high number of false negative and false positive results. Indeed, the absence of fecal occult blood at the time of the test does not exclude the presence of a CRC, because the mucous membranes can bleed intermittently. Furthermore, when the test gives a positive result, a further confirmation analysis must be carried out in order to exclude a false positive due to other benign lesions. In Italy, the Ministry of Health recommends performing this test, in individuals aged between 50 and 69, every two years [Howlader N NA. et al., 2013; Cannizzaro R., 2018].

In addition to the fecal occult blood test, endoscopic investigations represent other screening methods that allows the removal of a benign polyposis formation before their malignant transformation. In Italy, recto-sigmoidoscopy is a more well-tolerated screening method compared to colonoscopy, and it is recommended only once in life in individuals between 55 and 64 years [Winawer SJ et al., 1993; Cunningham D. et al., 2010; Clarke WT. et al., 2019; AIOM 2021].

Patients who are at high risk of developing CRC should undergo these screening methods more frequently. In particular, individuals with Lynch syndrome must undergo endoscopic checks every two years starting from the age of 25; this frequency is reduced to one year when the individual is over 40 years of age. This high frequency is due to the increased risk that patients with Lynch syndrome have of developing CRC in less than two years despite the absence of lesions observed with colonoscopy [Vasen HF et al., 1995; Järvinen HJ et al., 2000; National Comprehensive Cancer Network, 2015].

Even for patients with FAP, screening tests must be performed earlier compared to healthy individuals; in particular, the use of Gastro-duodenal endoscopy (GDE) allows to exclude the presence of duodenal cancer, which is represent the second common cause of death in FAP affected patients [Heiskanen I et al., 1999; Campos FG et al., 2015].

Despite the different screening methods previously described, the diagnosis of colorectal cancer is only performed with histopathological examination of a suspected lesion identified after surgical resection of the colon or during colonoscopy. To date, pancoloscopy is considered the gold standard for the diagnosis of colorectal cancer and can be performed after appropriate sedation. Although it is a very sensitive method (97% sensitivity and 98% specificity), several studies have described how after this procedure adverse events can occur; these are represented by colon perforation in 0.1% of cases, hemorrhage in 0.3% of cases and in the most serious cases patients' death (0.01-0.03%) [Byers T et al., 1997; De' Angelis N. et al., 2018; Kim SY et al., 2019]. In some cases, it is possible to use recto-sigmoidoscopy associated with CT colon with good sensitivity and specificity only limited to the first 60 cm of the anatomical district.

The use of imaging techniques is also very important for assessing the progress of the disease and for identifying metastasis located at a distance from the primary tumor site [Cunningham D. et al, 2010; AIOM 2021].

1.1.7 CRC Treatment: Surgical resection and pharmacological strategies

The choice of the correct therapeutic approach for CRC patients depends on several factors, including the evaluation of the prognostic marker levels such as the Carcino-embryo antigen evaluation (CEA) (also used in the follow-up of the patients), the age of the patient, the symptoms associated with the neoplasm, the anatomical location of the tumor and its stage.

As previously described, the stage of the tumor is essential for the choice of an adequate therapeutic approach; moreover, it is also considered a good prognostic factor. Indeed, several studies have highlighted that different factor such as the tumor extension, lymph node involvement and the presence of metastases can significantly affect the 5-years-survival rate; in this regard it can vary from a value of 90% in the earliest stages up to a value of 10% in the most severe cases [Chen K. et al., 2021].

The first therapeutic option for CRC is represented by surgical removal of the tumor tissue with adequate disease-free margins. When the cancer is at an early stage, surgery could represent a conclusive treatment. In some cases, in order to reduce the tumor mass before surgical treatment, a chemotherapy treatment can be carried out to; moreover, in the case of rectal cancer, surgical resection can also be associated with radiotherapy [Kuipers EJ. et al., 2015; Li Y. et al, 2016]. Despite the total resection of the tumor tissue through surgical treatment, about 35% of patients develop a recurrence of the disease which can occurs in the first three years in 80% of cases. Although local relapses are rare in colon cancer, resurgence of the disease is most found in the other sites such as liver, abdominal lymph nodes, peritoneum and lung. Furthermore, despite the presence of many screening programs, nearly a quarter of CRCs are diagnosed at an advanced stage [Keum N. et al., 2019]. In these patients the therapeutic approach includes the use of adjuvant chemotherapy protocol; in particular, the choice of the combination of drugs differs according to the stage of the tumor.

Chemotherapy treatment is based on the use of different chemotherapeutic drugs including 5-fluorouracil, capecitabine, irinotecan and oxaliplatin which can be used alone or in combination (5-FU/LV, CAPOX, XELOX FOLFOX, FOLFOXIRI, and FOLFIRI). The choice of the correct chemotherapy approach

depends not only on the stage of the tumor, but also on the characteristics of the patient [Xie YH. Et al., 2020; AIOM 2021]. For example, patients with good prognosis or intolerance to specific chemotherapy drug such as oxaliplatin, receive a treatment consisting in the combination of 5-FU and Folinic Acids. [Mamazza J et al., 1982; Schmoll HJ et al., 2012; Van Cutsem E. et al., 2008].
Another important factor is associated with different enzymes responsible for the metabolism of cytotoxic agents. If the activity of these enzymes is altered due to gene mutations, the treatment with chemotherapeutic agents may be associated with greater toxicity due to an increased levels of toxic metabolites in CRC patients compared to healthy individuals [Ruzzo A. Et al. 2014]. An example is represented by dihydropyrimidine dehydrogenase (DPD), an enzyme involved in the metabolism of fluoropyrimidines. In this regard, it is important to analyze allelic mutations associated with DPYD gene that could determine a reduced enzymatic activity of DPD with an increased toxicity in patients treated with fluoropyrimidines [Meulendijks D. et al., 2015; Lunenburg CATC. et al., 2016]. Therefore, it is recommended to perform the analysis of the following mutations: c.1236G>A, c.1679T>G, c.1905+1G>A, c.2846A>T and c.2194G>A [Amstutz U. et al., 2011; Boige V. et al., 2016; Ruzzo A. et al., 2017; European Medicines Agency (EMA); Henricks LM. et al., 2018; Del Re M. et al. 2019; Iachetta F. et al., 2019]. Another enzyme involved in the metabolic degradation of chemotherapy agents is uridine-glucuronosyl-transferase (UGT). It is involved in the hepatic metabolism of an active metabolite of irinotecan called SN-38, [Cortejoso L. et al., 2012]. Several studies have shown that the UGT1A1*28 allelic variant is associated with reduced metabolism of irinotecan, while individuals with the wild type allele (UGT1A1*1) could tolerate higher doses of irinotecan with a better therapeutic outcome [Toffoli G. et al., 2010].
In order to select the correct therapeutic approach for patients with advanced metastatic CRC, the evaluation of the mutational status of exons 2,3,4 of the RAS genes (KRAS and NRAS) and the mutations of the BRAF gene plays an important role [Douillard JU et al., 2013]. During first-line therapy, these patients can be treated with a FOLFOX/FOLFIRI chemotherapy in combination with anti-VEGF drugs such as bevacizumab [Masi G. et al., 2005; Grothey A et

al., 2008; AIOM 2021]. If mutational analysis reveals a RAS and BRAF WT status, FOLFOX/FOLFIRI protocol can be also combined with an anti-EGFR drug such as cetuximab; moreover, in some cases, cetuximab can also be used in a monotherapy protocol [Jonker DJ et al., 2007; Van Cutsem E et al., 2007; Amado RG et al., 2008; Karapetis et al., 2008].

If patients treated with first line therapy do not show a disease regression, it is possible to switch to a second line therapy which, based on the type of therapy previously performed, may include the use of other drugs such as Aflibercept (anti VEGF-A, VEGF-B and PlGF) in association with FOLFIRI [Van Cutsem E. et al., 2012] or the use of other molecules such as Regorafenib, a multi-kinase inhibitor also administered in the third line therapy [AIOM 2021].

In addition to gene alterations previously described the evaluation of MSI also plays an important therapeutic role. High-MSI (MSI-H) in metastatic CRC seems to be associated with chemoresistance phenomena [Innocenti F. et al., 2017]. Furthermore, the MSI-H seems to be related to an increased sensitivity to immunotherapy [Snyder A. et al., 2014; Rizvi NA. et al., 2015; Gelsomino F. et al., 2016; Rosenberg JE. et al., 2016; Yuza K. et al. 2017; Goodman AM. et al. 2017]. Several studies have shown the benefit of immunotherapy drugs such as pembrolizumab, nivolumab and ipilimumab in the treatment of metastatic CRC with MSI-H [Le DT. et al., 2017; Overman MJ. et al., 2017; Overman MJ. et al., 2018; Dung T Le. et al., 2020; Lenz HJJ. et al., 2020].

These results then allowed the Food and Drug Administration (FDA) to approve the use of these drugs. In particular, the use of pembrolizumab and the use of nivolumab alone or in combination with ipilimumab was evaluated for the treatment of metastatic colorectal cancer with MSI-H pretreated with standard therapies or also as a first line of treatment [Lee J Yothers G. et al., 2018; Chalabi M. et al., 2020; André T. et.al., 2020; AIOM 2021].

1.2 microRNAs

1.2.1 Biogenesis and mechanism of action

Non-coding RNAs (ncRNAs) are also involved in the mechanisms of epigenetic regulation. Among these, microRNAs (miRNAs) play an important role; they are small non-coding RNA strands of about 22 bp which are involved in different mechanisms. miRNAS were discovered around the '90s thanks to scientists Victor Ambros, Rosalind Lee and Rhonda Feinbaum who observed these molecules during experiments on the larval development of Caenorhabditis elegans [Lu H et al., 2010].

The biogenesis of miRNAs occurs through different steps. In the first step, a primary transcript RNA (pri-miRNA) transcribed by RNA polymerase II, undergoes the addition of a 5′ cap and a 3′ poly-A tail. Subsequently, thanks to the activity of a specific ribonuclease, called Drosha, associated with DGCR8, the pri-miRNA is processed into a small stem-loop structure of 55-70 nt. The pre-miRNA thus formed is exported to the cytoplasm by exportin-5, which is a member of the Ran-dependent nuclear transport receptor family. In this cellular compartment, pre-miRNA hairpin undergoes a cleavage by Dicer, another specific ribonuclease, with the help of TRBP (transactivating response RNA-binding protein), thus forming a mature miRNA duplex 19-22 nt in length [Cowden Dahl KD. et al., 2009; Rupaimoole R. et al., 2016; LaPierre MP. et al., 2017; Kotyla et al., 2020] which is then loaded onto RISC (RNA-induced silencing complex) and AGO2 (Argonaut 2 protein). Although only one of the two strands (the strand guide) is associated with the silencing complex, in some cases, the second strand can also act independently silencing other targets.

miRNAs, thanks to their sequence called seed sequence, are able to pair with 3'UTR region of messenger RNA (mRNA) of specific target genes; if the complementary is partial, there is only a reversible translational repression of mRNA target. On the other hand, when there is a perfect complementarity between miRNAs and the target sequence, the silencing complex degrades target mRNA; this effect determines a silencing of gene expression [He and Hannon, 2004; Doench et al., 2004; Scott GK. et al., 2007; Kappelmann M. et al., 2013].

The human genome encodes thousands of miRNAs which often exhibit a specific tissue distribution [Bentwich et al., 2005; Berezikov et al., 2005]; moreover, in more complex organisms, the number of transcribed miRNAs is higher than in simpler organisms [Lu Y et al., 2018]. Different studies have shown how these molecules are able to regulate different processes such as differentiation, cell growth and apoptosis [Cairns RA et al., 2011; Soga T, 2013; Carding S. Et al., 2015; Thursby E. et al., 2017; Romero-Cordoba SL et al., 2018]; therefore it appears possible their involvement in several pathologies and in particular in tumor development and progression [Hong YH et al., 2005; Lu j et al., 2005; Morrison DJ et al., 2016;]. Based on the function of the target gene, miRNAs can be divided into oncogenic miRNAs and tumor-suppressor miRNAs. Tumor-suppressor miRNAs interact with oncogenic genes; therefore, their levels are often downregulated in tumors compared to healthy tissue. For example, several studies have shown how miR-let-7 family is downregulated in several tumors including CRC, resulting in an increase in the expression of its RAS or MYC targets; another example is represented by miR-34a and miR-331-3p also downregulated in other type of cancers [Ali Syeda Z. et al., 2020]. Conversely, miRNAs with oncogenic function interact with tumor-suppressor genes; these miRNAs are often increased in different type of cancers. Indeed miR-21, directed against PTEN, appears to be up-regulated in different solid and haematological tumors [Kluiver et al., 2006]; in addition, miR-221 and miR-222 levels were also found to be increased in different types of cancer [Galardi S. et al., 2007; Mercatelli N.et al., 2008; Ali Syeda Z. et al., 2020]. Further studies have also highlighted a putative role of miRNAs in promoting change in cellular metabolism and in acquiring the ability to use alternative carbon sources. This would allow cancer cells to gain an advantage over normal cells and to maintain high proliferation rates even at low oxygen concentrations [Sanders ME, 2000; Mcfarland LV et al., 2006; Rastmanesh R. et al., 2011; Shamekhi et al., 2020].

1.2.2 Dysregulated miRNAs in CRC

Due to the involvement of different miRNAs in various neoplastic diseases, several studies have aimed to understand how these small non-coding RNA are also involved in the development of CRC. In particular, several studies have shown how different miRNAs can interact with the Wnt signaling pathway which appears to be a frequently altered pathway in many CRC cases. More in detail, it was observed how the overexpression of miR-135a/b can result in a downregulation of APC levels, thus inducing downstream Wnt pathway activity [Nagel R et al., 2008]. Furthermore, it has been shown that other miRNAs are also able to interact with the Wnt pathway. For example, mir-34a/b/c seems to play a role as tumor suppressor miRNAs; indeed, through their interaction with WNT1, WNT3, LRP6, β-catenin and LEF1 they are able to negatively regulate the Wnt pathway. [Kim NH et al., 2011].

Lv S. and colleagues showed that miR-520e, through the interaction with the oncogene AEG-1, is able to suppress the proliferation and invasion of colorectal cancer cells regulating Wnt/GSK-3β/β-catenin signaling pathway [Koni M. et al., 2020; Lv S. et al., 2020].

Other studies have shown that miRNAs perform their action also interacting with other altered pathways in CRC such as the MAPK pathway. For example, miR-31 appears to positively regulate KRAS levels by interacting with RASA1, a KRAS inhibitor [Kent OA et al., 2016].

Furthermore, other evidence has suggested a possible role of miR-425-5p in the pathogenesis of KRAS-mutated CRC [Angius A. et al., 2019]. An alteration of the MAPK pathway may also depend on alterations in the BRAF gene. Some studies have shown that miR-31 also could be associated to BRAF mutations and to a greater cancer aggressiveness in CRC [Nosho K et al., 2014; Ito M et al., 2014; Choi YW et al., 2016]. Furthermore, Wang and colleagues showed that miR-378-5p suppresses cell proliferation and induces apoptosis in CRC cells by targeting BRAF [Wang Z et al., 2015].

Other miRNAs were found dysregulated in the CRC; for example 12 miRNAs (miR-7, -17, -20a, -21, -92a, -96, -106a, -134, -183, -196a, -199a-3p, and -214) showed higher expression levels in stool samples from CRC patients compared

to healthy controls, whereas eight miRNAs (miR-9, -29b, -127-5p, -138, -143, -146a, -222, and -938) were shown to be downregulated [Ahmed FE. et al., 2013 Mármol I. et al., 2017]. Different studies have focused their attention on the miRNAs as these could function as potential diagnostic and prognostic biomarkers for different types of cancer including CRC. In particular, the analysis of the circulating miRNAs could represent the starting point for the development of accurate and non-invasive screening method for early CRC detection.

1.3 Gut microbiota

1.3.1 Gut microbiota and cancer

The development of tumors is associated with several genetic and epigenetic factors strongly modulated by the exposure to several environmental stimuli. The cumulative action of several factors towards the development of tumors has been defined as exposome, i.e. the cumulative effects of all environmental factors (e.g., diet, drugs, exposure to infectious agents, UV radiation, environmental toxins, pollutants, behavioral and socio-economic factors) and the related biological and molecular responses which occur during the entire life of individuals [Miller GW. et al., 2014]. The exposome is able to induce tumor formation through multiple mechanisms mainly associated with the dose, time and duration of exposures, the combination between individual factors and susceptibility with environmental risk factors and the interaction between environmental risk factors and specific individual genetic and epigenetic alterations [Juarez PD. et al., 2018].

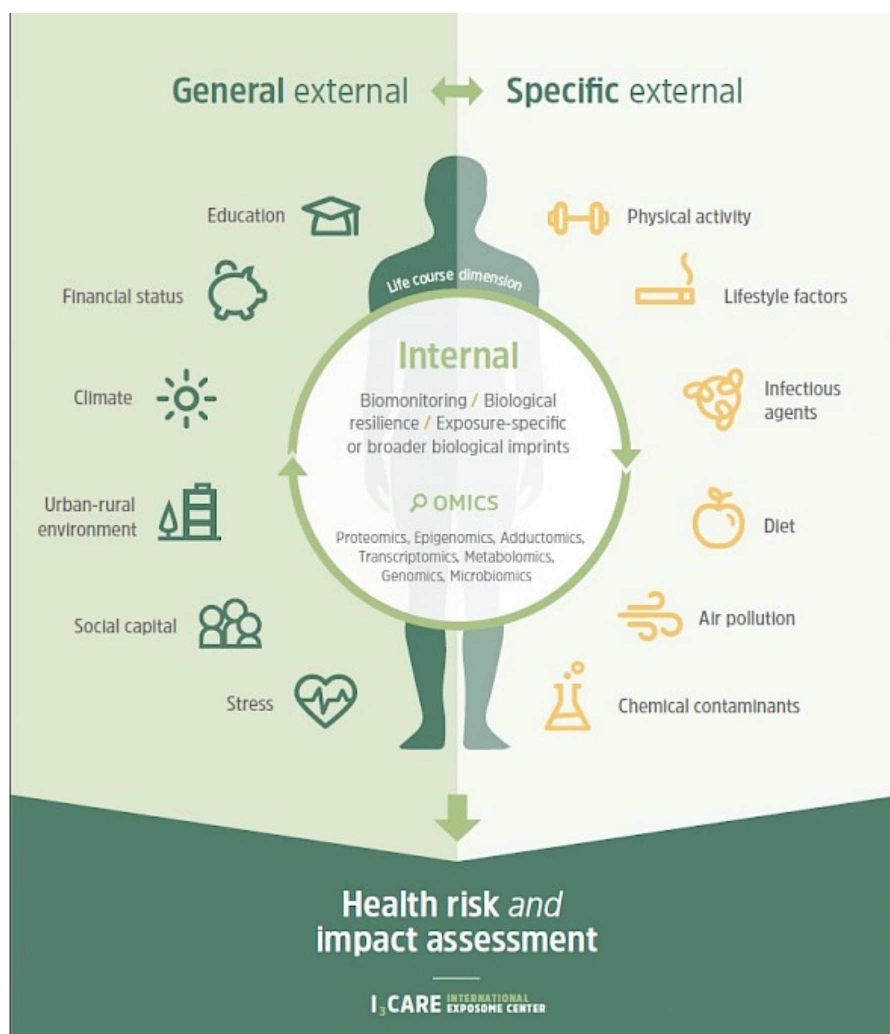

Figure 1. Schematic representation of human exposome. Human exposome is influenced by specific external environmental factors, general exposure to environmental stimuli and internal modifications related to the two aforementioned external triggers. Overall, these stimuli influence the health status of individuals modifying the risk of cancer development. Image from https://exposome.iras.uu.nl/what-is-the-exposome/

It was recently demonstrated, how human exposome is profoundly modulated by human microbiota, i.e. all the microorganisms which colonize the exposed surfaces of the human body by influencing different physiological and pathological processes.

The composition of the human microbiota is strongly associated with the health status of the host; indeed, the alteration of human microbiota homeostasis, also defined dysbiosis, is associated with different diseases, including cancer [Wang B. et al., 2017]. In this context, it was demonstrated as some microorganisms play a detrimental role for the host while others are associated with protective

effects. All these microorganisms compose the human microbiota which is an active part of the human exposome [Siroux V. et al., 2016].
Of note, human microbiota can be distinguished into small subgroups depending on the human surface considered. Therefore, we can have the oral microbiota, gut microbiota, vaginal microbiota, and so on. The gastro-intestinal or gut microbiota is the most characterized and studied as it strongly influenced the host homeostasis [Lin L. et al., 2017]. The gut microbiota is composed by different populations of microorganisms that colonize the entire gastro-intestinal tract, in particular the large intestine. These microorganisms include (bacteria), but also viruses, fungi and archaea. Throughout the entire lifespan the individuals are constantly exposed to the gut in a strong relationship where the gut microbiota influences the human host and the human host influences the gut microbiota [Vivarelli S. et al., 2019].
The gut microbiota is involved in different physiological functions mainly represented by the modulation of host metabolism, the maintenance of the intestinal barrier, the metabolism of xenobiotics, protection against pathogenic microorganisms, modulation of the host's immune system (Figure 2) [Dieterich W. et al., 2018].

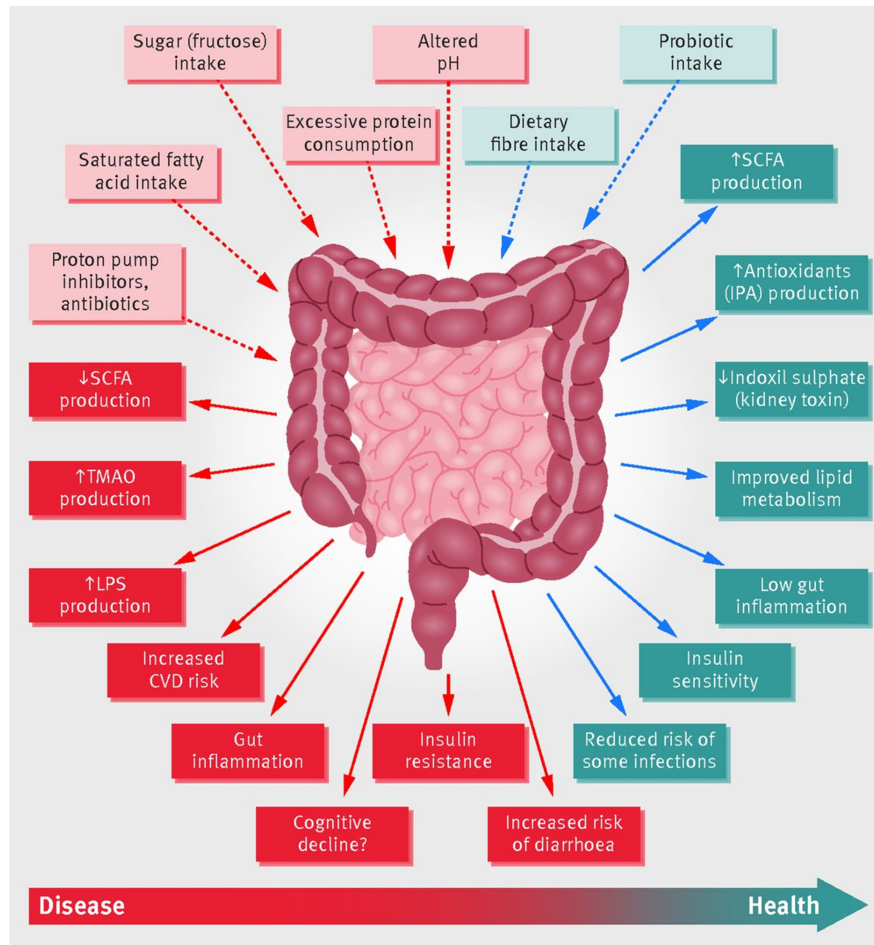

Figure 2. Main functions of gut microbiota [Valdes AM. et al., 2018].

More in detail, some commensal bacteria produce essential micronutrients such as K and B vitamins which have positive effects on human health. Furthermore, some of them are capable of transforming amino acids into signal molecules (e.g., glutamine to gamma-amino butyric acid, GABA; histidine to histamine). Finally, some bacteria are able to secrete short chain fatty acids (SCFAs), which derive from the bacterial fermentation of dietary fibers. SCFAs are very important because, once absorbed in the intestine, they are conveyed to the liver where they are used as a fundamental energy resource. Furthermore, SCFAs are able to control carbohydrate and lipid metabolism [Visconti A. et al., 2019].

Different from gut microbiota is the gut microbiome, that is the set of all the genomes of intestinal microorganisms encodes a number of genes 100 times greater than those encoded by the human genome. In the last 10 years, the advent of metagenomics, combined with next-generation sequencing (NGS) and the analysis of rRNA16S amplicons, have allowed us to characterize, at the species

level, both the abundance and the diversity of the intestinal microbiome [Nichols RG. et al., 2020]. Studies of metagenomics, together with those of transcriptomics and metabolomics are helping to reconstruct the real impact of the architecture of the microbiome on human health (Figure 3) [Gomaa EX., 2020]. A recent study has made it possible to identify 4,644 intestinal prokaryotic species, which can be used as a future reference for metagenomic studies [Almeida A. et al., 2021].

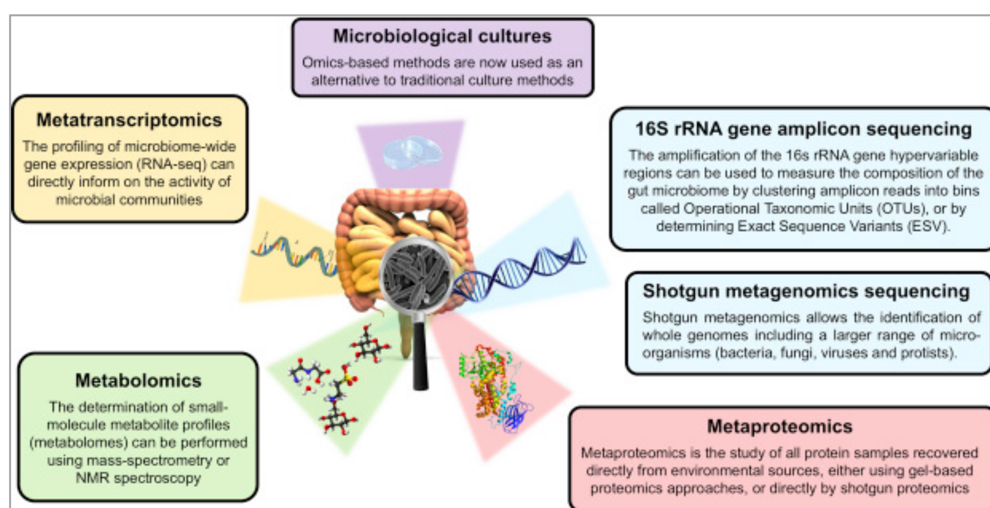

Figure 3. Molecular and proteomics techniques for the analysis of gut microbiota and microbiome [Mesnage R. et al., 2018].

As already mentioned, there is a two-way relationship between the intestinal microbiota and the human host, therefore variables related to the host (such as diet, and, more generally, lifestyle) influence the composition of the intestinal microbiota, and *vice versa* [Vujkovic-Cvijin I. et al., 2020]. Metagenomic analyses have facilitated the identification of intestinal bacterial species that are directly involved in the etiology of diseases that afflict humans, such as cancer. Among the diseases that are directly associated with intestinal dysbiosis there are tumors, both gastrointestinal and tumors that have developed in sites distant from the digestive system [Vivarelli S. et al., 2019]. Currently, the number of studies that have demonstrated the correlation between the intestinal microbiota and human diseases has increased exponentially. All these studies underline how the modulation of the intestinal microbiota composition can be an adjuvant approach to anti-tumor therapies [Kho ZY. et al., 2018]. In this context, the European Union is funding an increasing number of research projects on the

human microbiota [Hadrich D., 2020]. One of the most important is the ONCOBIOMA project, which is currently exploring the functional role of the microbiome in the tumorigenesis of four different cancers: breast, colon, lung and skin melanoma [Hadrich D., 2020; ONCOBIOME Project]. This project will characterize the functional interconnection existing between the composition of the intestinal microbial population, metabolism and host immunity in more than 9,000 cancer patients enrolled in 10 different Countries. This will allow us to identify the so-called Gut Onco-Microbiome Signature (GOMS), that is, the set of features of the intestinal onco-microbiome. This signature will then be correlated to tumor frequency, prognosis and progression, as well as to the therapeutic response of the cancer patient [Hadrich D., 2020; ONCOBIOME Project].

In line with the aim of the ONCOBIOMA project, a recent groundbreaking research has shown that the intestinal microbiome has modulatory effects on the host's immune response, tumorigenesis and tumor development, and response to anti-cancer therapy. In this sense, the ONCOBIOMA project will allow to generate a database of the gut microbiome of the cancer patient able to help predict the risk of developing cancer and to customize cancer therapy based on the specific individual composition of the microbiota, in terms of both diversity and abundance of microorganisms populating the gastrointestinal tract [Vivarelli S. et al., 2019 WAJS]. The knowledge that will be generated by such analyses will allow the development of real adjuvant strategies capable of improving the therapeutic outcome of cancer patients based on the analysis of gut microbiota and its enrichment through fecal transplantation or the administration of probiotics [Vivarelli S. et al., 2019 WASJ].

More recently, other studies have demonstrated the existence of the so-called miRNA-microbiota axis through which the gut microbiota affects the health of the host. This is a two-way interaction mechanism, as the host's miRNAs modulate the gut microbiota and *vice versa* [Zhao Y. et al., 2021]. In the case of colorectal cancer, it has been observed that *Fusobacterium nucleatum* can aggravate host chemo-resistance through the down-regulation of miR-18a and miR-4802. Conversely, miR-515-5p and miR-1226-5p, if over-expressed in the

host, can promote the proliferation of pathogenic bacteria (i.e., *Fusobacterium nucleatum* and *Escherichia coli*) which have been shown to be associated with neoplastic transformation of colocytes [Li M. et al., 2020]. The characteristics of the miRNA-microbiota axis are summarized in Figure 4.

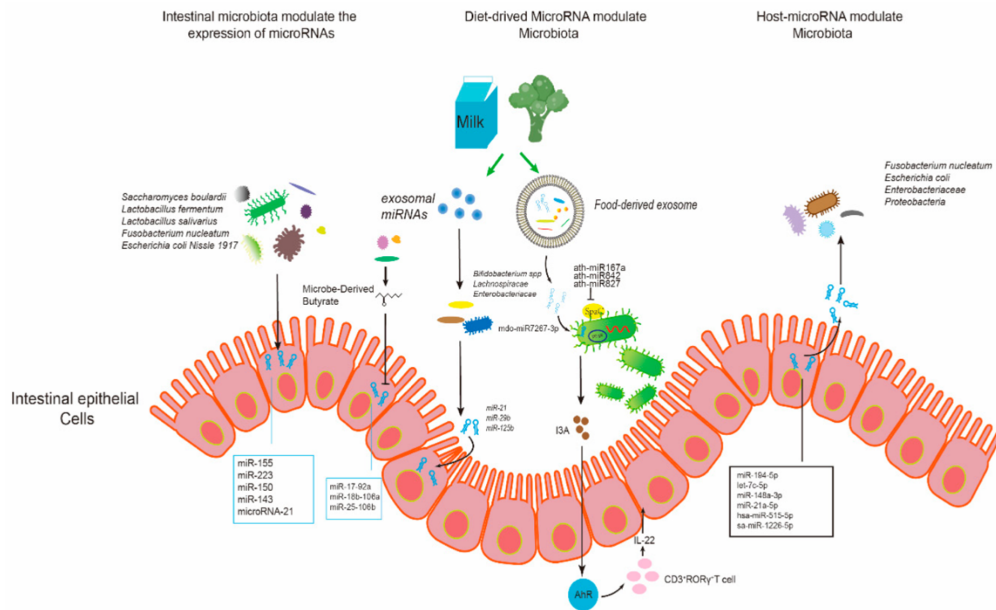

Figure 4. Relationship existing between miRNAs and gut microbiota and their effects on gene regulation [Bi K. et al., 2020].

For all the mechanisms listed above, the maintenance of intestinal balance, or eubiosis, helps to positively modulate the metabolic functions of the host. Any imbalance of this eubiosis, i.e. gut dysbiosis, is associated with the onset of multiple diseases, including tumors [Shelfin AM. et al., 2014].

1.3.2 Involvement of Gut microbiota in neoplastic transformation mechanism

Metagenomics and metabolomics studies suggested that the gut microbiota can play both a pro-tumor and anti-tumor role depending on the abundance and diversity of the microbial population [Fulbright LE. et al., 2017]. It has been well-established that the development of tumor (both intestinal and other forms of cancer) modulates the gut microbiota; on the opposite, the composition of the intestinal microbiota strongly affects tumorigenesis and cancer progression [Vivarelli S. et al., 2019].

Many intestinal bacteria which are normally present in the gut are capable of producing molecules with proven anti-cancer effects. For example, SCFAs (such

as butyrate and propionate) produced by some intestinal bacteria inhibit the histone deacetylases of cancer cells, thus negatively interfering with the proliferation mechanisms of these cells as demonstrated in colorectal cancer and lymphoma [Jan G. et al., 2002; Wei W. et al., 2016].
Furthermore, certain molecules produced by some species of intestinal bacteria can directly stimulate the host's immune system to counteract tumor growth. For example, bacterial lipopolysaccharide (LPS), the major component of the outer wall of gram-negative bacteria, activates the pattern recognition receptor (PRR) toll-like receptor 4 (TLR4) of immune cells which in turn cytotoxic T-cell [Paulos CM. et al., 2007]. Similarly, the mono-phosphoryl lipid A (MPL) from *Salmonella enterica* was tested as a potential adjuvant in the cervical cancer vaccine formulation [Paavonen J. et al., 2009]. In addition, pyridoxine (vitamin of group B) produced by some bacteria is able to directly stimulate the immune-surveillance mechanisms activated by the host against cancer cells [Aranda F. et al., 2014].
From such observations, some bacteria are now defined "probiotic" (pro-life). In fact, probiotics are defined by the Food and Agriculture Organization (FAO) as "live organisms which, when consumed in adequate quantities, confer a benefit to the health of the host" [Hill C. et al., 2014]. It has been observed that many commensal bacteria act as probiotics thanks to their ability to protect the host from disease and in general to have positive effects on human health, protecting against the development of intestinal dysbiosis and increasing the immune defense mechanisms against pathogens (and against cancer cells; Figure 5) [Fulbright LE. et al., 2017].

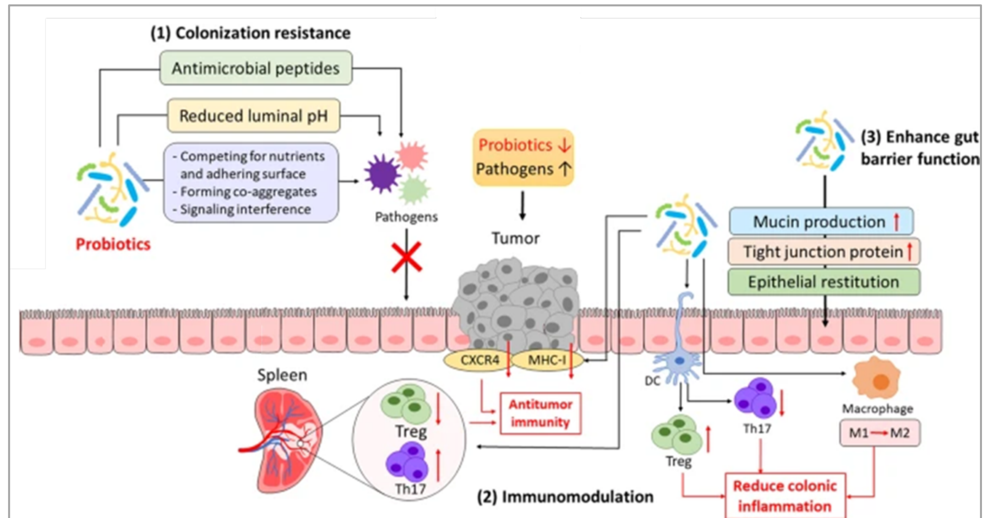

Figure 5. Probiotics maintain gut eubiosis ameliorating colorectal cancer patients' health statuts. The protective action of probiotics is mediated by three mechanisms: (1) inhibition of pathogen colonization; (2) positive immunomodulation; (3) improvement of gut barrier [Fong W. et al., 2020].

For example, the administration of Mutaflor (*Escherichia coli* Nissle 1917) in combination with the antibiotic Rifaximin significantly increases the anti-inflammatory effect of the antibiotic administered alone in patients with inflammatory bowel disease or inflammatory disease [Dembiński A. et al., 2016].

Many probiotics have been shown, in various preclinical studies, to possess anti-neoplastic activity. For example, the ferrichrome produced by *Lactobacillus casei* induces apoptosis of tumor cells through direct modulation of the JNK pathway [Konishi H. et al., 2016]. Instead, *Lactobacilli* can stimulate the immune system, including natural killer (NK) and dendritic cells (DC), to induce a TH1 anti-tumor response resulting in the elimination of pre-cancerous and cancerous cells, however, the precise bacterial determinants exerting these immunostimulant effects have not yet been identified [Baldwin C. et al., 2010]. The anti-tumor effects of the gut microbiota are summarized in Figure 6.

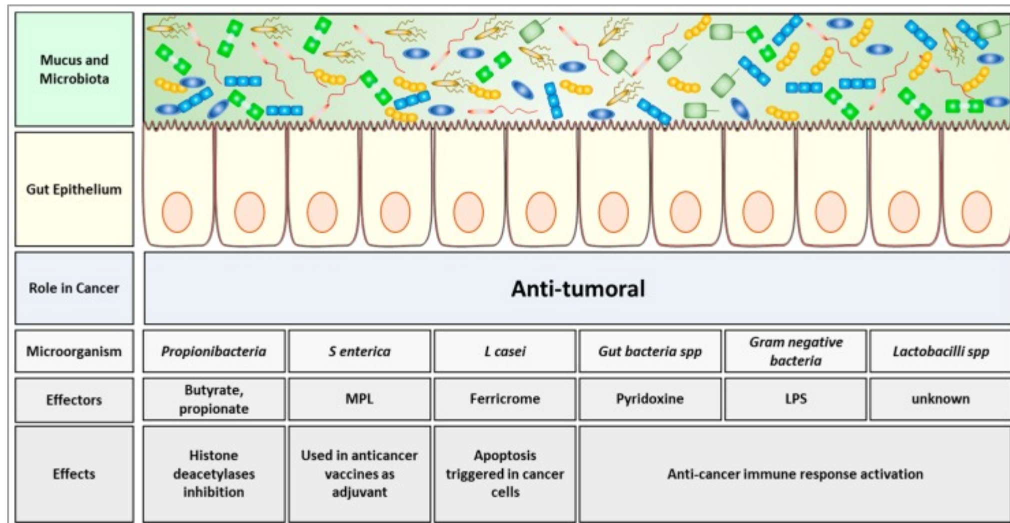

Figure 6. Antitumor effects of gut microbiota. [Vivarelli S. et al., 2019].

Other intestinal bacteria are known to have a tumor-promoting effect either through the production of toxic metabolites/catabolites or through direct interaction with the tumor [Sheflin AM. et al., 2014]. In general, gut dysbiosis favors the proliferation of pathogenic bacteria that are often responsible for these toxic effects that contribute to the onset of various inflammatory bowel diseases, and finally cancer [Lane ER. et al., 2017; Caputi V. et al., 2018; Rea D. et al., 2018].

Pre-clinical studies using germ-free mice (with sterile intestines) have shown that there are many mechanisms through which pathogenic bacteria can favor tumor development [Nougayrede JP., 2006; Arthur JC. et al., 2012]. For example, *Helicobacter pylori* produces the toxin CagA which has been shown to directly stimulate tumor transformation typical of gastric cancer [Hatakeyama M., 2017]. Although a large number of bacteria have been identified as pro-tumor, at present only *Helicobacter pylori* is considered a Class I carcinogen by the WHO [Toller IM. et al., 2011; Grasso F. et al., 2015; Moss SF., 2017].

During intestinal infections and in the case of dysbiosis, many pathogenic bacteria produce toxins capable of producing direct damage to the DNA double helix, contributing to genomic instability and the initiation of neoplastic transformation [Halazonetis TD., 2004; Frisan T., 2016]. For example: colibactin (CBT) or cytolethal distending toxin (CDT) both produced by *Escherichia coli* have nuclease activity. Once released into the intestinal

epithelium, they actively penetrate epithelial cells where they induce double-strand breaks (DSB) in the cellular genomic DNA, promoting a transient arrest of the cell cycle and favoring the appearance of possible mutations during the stages of repair, and therefore helping tumor transformation [Lara-Tejero M., 2000].

Intestinal pathogens can also directly interfere with DNA repair pathways. For example, the inositol-phosphate phosphatase (IpgD) or the A cysteine-protease (VirA) produced by *Shigella flexneri* are able to induce the degradation of the anti-apoptotic protein p53, increasing the likelihood of genomic DNA mutations occurring in the course of replication cycle of the affected cell [Bergounioux J. et al., 2012]. Similarly, CagA produced by *Helicobacter pylori* induces proteasome-mediated degradation of p53 in gastric epithelial cells, interfering with the Akt pathway and thus promoting tumor transformation in these cells [Buti L. et al., 2011].

Furthermore, CagA of *Helicobacter pylori*, FadA of *Fusobacterium nucleatum* and the MP toxin produced by *Bacteroides fragilis* interact directly with the E-cadherin of the epithelial cells of the gastro-intestinal tract and activate the pro-proliferative signals mediated by activated β-catenin, thus promoting uncontrolled cell proliferation [Murata-Kamiya N. et al., 2007; Wu S. et al., 2007; Rubinstein MR. et al., 2013]. *Helicobacter pylori* CagA also activates the pro-survival pathways of MAPK and AKT in target cells [Kuijl C. et al., 2007; Bronte-Tinkew DM. et al., 2009].

Another mechanism by which intestinal bacterial derivatives can promote cancer is by promoting oxidative stress [Ding SZ. et al., 2007; Wada Y. et al., 2018]. Furthermore, many of these bacteria are able to induce local inflammation and inhibit the host's immune response directed against cancer cells, thus promoting cell proliferation on the one hand and tumor immuno-suppression on the other [Belkaid Y. et al., 2014].

Finally, some bacteria interfere with the host's hormonal metabolism. For example, the secretion of β-glucuronidase by *Clostridium leptum* or *Clostridium coccoides* locally increases the bioavailability of the host's estrogen hormones, which can activate estrogen receptors in target cells, promoting proliferation

(particularly in cells of the endometrium and mammary gland) [Plottel CS. et al., 2011]. The pro-tumor effects of the gut microbiota are summarized in Figure 7.

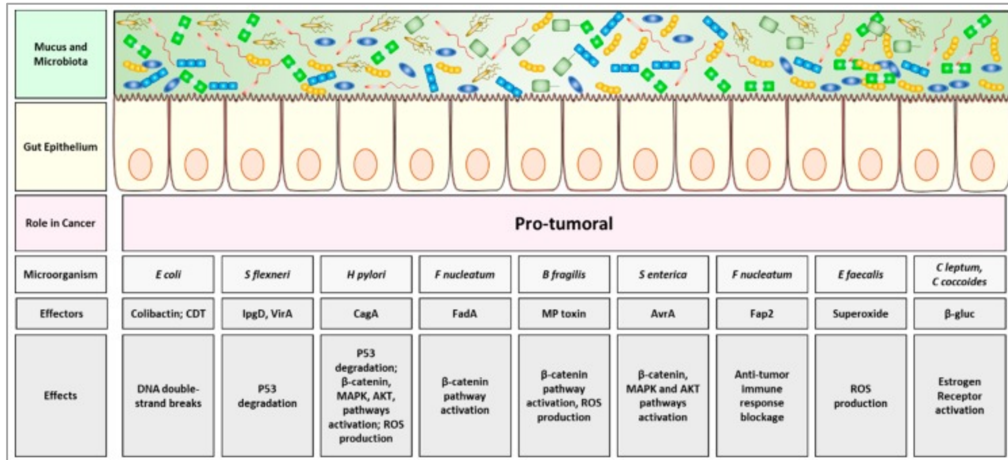

Figure 7. Pro-tumor effects mediated by gut microbiota. [Vivarelli S. et al., 2019].

Despite the advancements in anticancer research, it is still difficult to determine whether a given microbiota or intestinal microbiome (in terms of quantity and quality of microorganisms present) can negatively or positively influence the development and progression of tumors [Kilkkinen A. et al., 2008]. For this reason, currently, several pre-clinical and clinical studies are trying to deeply understand the pro-tumor and antitumor mechanism mediated by gut microbiota. In addition, it is necessary to keep in mind the importance that the host's behaviors and habits, throughout their entire existence and how diet and other environmental factors influence the homeostasis of gut microbiota [Conlon M. et al., 2014].

1.3.3. Involvement of Gut Microbiota in Colorectal Cancer

The group of bacteria, archaea and eukarya composing gut microbiota has co-evolved with the host over thousands of years to form an intricate and mutually beneficial relationship. It has been estimated that the number of microorganisms colonizing the gastrointestinal tract is about 10 times more than the number of human cells and the genomic content (microbiome) exceeds human genome by over 100 times.

The formation and evolution of gut microbiota start during the birth when the gastrointestinal tract is rapidly colonized. The mode of delivery appears to affect the microbiota composition, with vaginally delivered infants' microbiota

containing a high abundance of *Lactobacilli* during the first few days, a reflection of the high load of these bacteria in the vaginal microbiota. In contrast, the microbiota of infants delivered by C-section is depleted and delayed in the colonization of the *Bacteroides* genus, but colonized by facultative anaerobes such as *Clostridium* species.

As already mentioned, different studies about microbiota demonstrated that the composition of the intestinal microbiota varies substantially amongst individuals and could vary also depending on genetic, pathologies, environmental and lifestyle factors. Among all the bacteria, Bacteroidetes and Firmicutes dominate with Proteobacteria, Actinobacteria, Fusobacteria, Spriochaetes, Verrucomicrobia.

Through their metabolic, nutritional, physiological and immunological functions, gut microbiota plays a key role as modulators of human diseases, especially gastrointestinal disorders including CRC. Indeed, gut microbiota cooperates with the host's defenses and the immune system to protect against pathogen colonization and invasion as well.

The microbiota contributes to the maintenance of gut homeostasis and to the protection against CRC through other important metabolic processes, such as amino acid modification, secondary biliary acids, short chain fatty acids (SCFA). SCFA are the primary end products of fermentation of non-digestible carbohydrates that become available to the gut microbiota. The major products are formate, acetate, propionate and butyrate. SCFAs are very important for the organism. Butyrate appears to play an important role in regulating the integrity of the epithelial barrier; it is the preferred fuel utilized by colonocytes and has been shown to enhance intestinal barrier function through increased expression of claudin-1 and Zonula Occludens-1 (ZO-1) and occludin redistribution proteins which are critical components of the tight junction assembly [Hajjar R. et al., 2020]. Moreover, increased circulating SCFAs are associated with reduced adipocyte lipolysis and adipogenesis and inhibition of insulin stimulated lipid accumulation in adipocytes via FFAR 2 signaling [Kimura I. et al., 2013; Gomes SD. et al., 2020].

Beyond its implication in metabolism and structural function, the microbiota performs an important role in the immune system. The interaction between immune system and gut microbiota has a central role in the maturation of immune system and response to self-antigens during the whole life; thus it has been suggested that dysbiosis may play a role in the development of diseases characterized by immune deregulation such as allergies, autoimmune, and inflammatory disorders and cancer.

Again, SCFAs play a fundamental role in the modulation of immune systems by interacting with several receptors expressed in the immune cells or modifying immune stimulating or immune modulatory factors [Carretta MD. et al., 2021; Corrêa-Oliveira R. et al., 2016].

More in detail, SCFAs interact with GPR109A/HCA2 receptor by inducing immune tolerance and Tregs maturation. Other, immune modulation properties are mediated by the inhibition of histone deacetylases (HDAC) induced by butyrate. Such inhibition further improves Tregs development and function. Beyond SCFAs, microbiota produces other metabolites from digested food that have important immunomodulatory functions as indole derivatives and polyamines.

All these interactions between microbiota and host environment can significantly alter several pathophysiological processes including infection, inflammation and diseases, including cancer.

As regards cancer, intestinal microbiota and its metabolic products may affect metabolic activation or inactivation of dietary phytochemicals, metabolism of hormones and the generation of tumor-promoting secondary bile acids, as well as inflammatory factors regulating tumorigenesis through metabolic activation and inactivation of carcinogens [Gagnière J. et al., 2016].

A perturbation of gut microbiota composition can induce a pro-inflammatory status with detrimental effects on human cells. In a dysbiosis state, an increased production of pro-inflammatory cytokines, including TNF, IL-1 and IL-17, has been observed. Such pro-inflammatory state triggers the activation of TLRs and the NF-kB signaling pathway which leads to uncontrolled cell proliferation and

the increase of angiogenesis processes, all mechanisms responsible for oncogenesis [Peng C. et al., 2020].

Different studies have highlighted how the alterations of the intestinal microbiota can be associated with an increased risk of CRC due to the activation of tumor-promoting mechanisms revealing how specific microbiota species are more associated with the presence of tumor with the formation of distinctive microbiota or a microbiological signature of tumor.

The metabolic processes mediated by gut microbiota are fundamental in the activation and detoxification of carcinogens, however, it also expresses many enzymes (such as beta-glucosidase, beta-glucuronidase, nitroreductase and alcohol dehydrogenase), directly involved in the development of certain neoplastic forms, in particular CRC.

Studies performed on germ-free mice demonstrated that the exposure to carcinogens did not result in the formation of intestinal tumors, while the use of the active metabolite of these substances lead to the formation of tumors, suggesting how some bacterial species may activate and produce carcinogens through their metabolism [Vannucci L. et al., 2008].

Some bacteria, including Clostridia, are able to produce beta-glucosidases involved in the production of toxic metabolites including 2-amino-3 methyl-imidazo [4,5-f]quinoline (IQ), which develop during the cooking process of red meat (a known risk factor for RCC). These activated compounds are capable of forming DNA adducts and therefore have a mutagenic effect on colonocytes by increasing the risk of neoplastic transformation [Bashir M. et al., 1987; Carman RJ. et al., 1988].

Another possible microbiota-related mechanism responsible for CRC development is the synthesis of mutagenic substances, such as fecapentenes produced by Bacteroides, ROS produced by *Enterococcus faecalis* or hydrogen sulphide produced by sulphate-reducing microorganisms, which cause DNA damage and induce neoplastic transformation [Strickertsson JA. et al., 2014; Hangi F. et al., 2019; Nguyen LH. et al., 2020].

Contrariwise, some commensal bacteria such as Bifidobacteria and Lactobacilli have an opposite role on these agents and antagonize their mutagenic effects by inactivating, these substances [Ding S. et al., 2020].

Currently, different studies are trying to characterize the individual microbial species that induce gut homeostasis perturbations responsible for an increased risk of cancer development. Such studies will allow the development of microbiota-based therapeutics based on the administration of specific bacteria, like probiotics, to enrich the gut microbiota and counteract pro-tumor phenomena [Banna GL. et al., 2017; Aghamajidi A. et al., 2022; Li J et. al,. 2022].

2. AIM OF THE STUDY

At present, the diagnosis and management of colorectal cancer (CRC) is still limited to invasive and poorly tolerated imaging techniques, including coloscopy and recto-sigmoidoscopy, while no effective circulating biomarkers have been validated for the early diagnosis of CRC. Different studies have demonstrated how several factors are involved in the development and progression of CRC and can be used for diagnostic purposes. Among these, epigenetic modifications and microbiota composition seem to be predictive for the development of CRC, however, the specific miRNAs with diagnostic potential or the microbiota factors associated with the modulation of miRNAs and the development or protection of CRC have not been clarified yet.

On these bases, the aim of the present study was to further establish the potential diagnostic and prognostic role of miRNAs in CRC as well as the impact of gut microbiota and its modulation in the expression levels of miRNAs known to be involved in the development and progression of this tumor.

For these purposes, computational and in vivo investigations on miRNA expression datasets and clinical samples obtained from CRC patients were performed in order to validate the diagnostic and prognostic potential of miRNAs in a pilot cohort of colorectal cancer patients and healthy controls and to establish the epigenetic modulation induced by *Lactobacillus rhamnosus GG* microbiota enrichment on the expression levels of the selected miRNAs.

3. MATERIALS AND METHODS

3.1 Computational identification of microRNAs involved in the development and progression of colorectal cancer and modulated by probiotics interventions

To preliminarily select a set of miRNAs strongly dysregulated and involved in the development and progression of CRC, several bioinformatic analyses were performed by consulting two of the biggest worldwide databases, GEO DataSets and TCGA.

First the microarray miRNA expression datasets related to CRC and contained in the GEO DataSets database and referred to colorectal cancer samples were selected to subsequently perform differential analyses between the expression levels of miRNAs in CRC patients vs normal control.

To select the most relevant miRNA expression datasets, the following search terms were used: “(("non-coding RNA profiling by array"[DataSet Type]) AND colorectal cancer) AND "Homo sapiens"[porgn:__txid9606]).

To strengthen the selection of datasets, the following inclusion and exclusion criteria were adopted:

Inclusion criteria:

- Datasets containing at least 30 samples (both normal and tumor samples);
- Datasets containing miRNA expression data of CRC patients and healthy controls;

Exclusion criteria:

- Datasets containing only normal or only tumor samples;
- Datasets containing miRNA expression data of animal models or human cell lines;
- Datasets with annotation information not available

The miRNAs expression levels contained in the selected datasets were analyzed using the GEO2R tool already available within the GEO DataSets database. This software, after normalizing the data contained in the dataset, performs a differential analysis of the expression levels of miRNAs between CRC patients and healthy controls, expressing the data as a base 2 logarithm of the fold change (log2FC). Among the miRNAs found to be deregulated between cases and

controls, only those with a p-value p <0.01 were considered; finally, the selected miRNAs were subsequently annotated using the latest nomenclature available published by miRBase (miRBase V 22) (http://www.mirbase.org/).

This approach was performed for all selected datasets. Subsequently, the dysregulated miRNAs (p <0.01) obtained from each dataset were merged using the Venn Diagrams tool of the Bioinformatics & Evolutionary Genomics (BEG) (http://bioinformatics.psb.ugent.be/webtools/Venn/) in order to select only those miRNAs that were found to be simultaneously strongly dysregulated in at least three of the analyzed datasets. Finally, from the data generated following the merge previously performed, only the 10 most up-regulated miRNAs and the 10 most downregulated miRNAs in patients with CRC compared to healthy controls were selected.

The differential expression of the levels of these 20 strongly dysregulated in CRC was showed as a heatmap where the blue boxes indicate the down-regulated miRNAs while red boxed the over-expressed ones.

As regards the analysis of datasets containing the expression levels of miRNAs before and after the administration of probiotics, the following search terms were used for the download of data matrices from GEO DataSets:

“(("non coding rna profiling by array"[DataSet Type]) AND gut microbiota) AND "Homo sapiens"[porgn:__txid9606]”.

Thus, miRNA expression datasets in colonic mucosa samples before and after administration of probiotic strains or fecal transplantation were selected.

Differential analyses were performed for all the selected datasets in order to identify miRNAs with altered expression levels in tumor versus healthy tissue and in colonic mucosa treated with probiotics versus untreated colonic mucosa.

To further confirm the data obtained through the analysis of microarray miRNA expression datasets, a further miRNA selection was performed by analyzing the expression levels of miRNAs in colorectal cancer samples and normal colon and rectal mucosa contained in the TCGA Pan-Cancer (PANCAN) database.

The data were downloaded by using the UCSC Xena Browser (https://xenabrowser.net) of the University of California Santa Cruz. For the analysis of miRNAs differentially expressed in tumor samples versus normal

controls the data reported on the "miRNA mature strand expression - Batch effects normalized miRNA data" dataset was considered. Of note, this dataset contains the miRNA expression data of a total of 10,188 samples obtained from different tumors (No. 9,534) and matched normal samples (No. 654) thus representing the more reliable cohort of cancer patients currently available on bioinformatics platforms. As regards colon and rectal cancer samples, the dataset contains 631 tumor samples and 101 solid normal tissues, however, the miRNA expression data were available only for 342 CRC samples and 11 normal mucosa.

The data downloaded from the TCGA database were manually analyzed by performing differential analyses on Excel. No normalization was needed as the TCGA data are already normalized. To strengthen the analysis and to select only representative miRNAs, a p-value of $p<0.01$ and a Log2FC value of $\pm$ 1.5 were adopted for the selection of significantly up-regulated and significantly down-regulated miRNAs.

3.2 Association between the most dysregulated miRNAs and the Main Genes Altered in Colorectal Cancer

In order to effectively evaluate the involvement of the selected miRNAs in the pathogenesis of CRC, the interaction levels between miRNAs and some of the most relevant genes altered in CRC were investigated.

For this purpose, a further computational analysis was performed by consulting the Catalog of Somatic Mutation in Cancer (COSMIC) (http://cancer.sanger.ac.uk/cosmic). Through this analysis, it was possible to obtain a list of the 10 most mutated and altered genes in CRC. Subsequently, the interaction levels between the previously identified dysregulated miRNAs and the 10 selected genes were then obtained by using the bioinformatics prediction tool microRNA Data Integration Portal (mirDIP - V 4.1.1.6,) (http://ophid.utoronto.ca/ mirDIP), a software able to integrate the bioinformatics prediction data contained in 26 different databases of miRNAs-mRNA interaction. These analyses allowed us to correlate the 10 most up-regulated and the 10 most down-regulated miRNAs with the selected most mutated and altered genes in CRC. The data obtained from the mirDIP software

allowed to categorize the interaction levels between the miRNAs and the targeted genes into 4 levels: very high, high, medium and low.

3.3 Functional role of selected miRNAs and modulated molecular pathways

To establish the functional role of the selected miRNAs with particular reference to CRC, further computational analyses were performed by using pathway prediction tools. In particular, the bioinformatics prediction tool DIANA-mirPath (v.3) was used to evaluate which cellular and molecular pathways were modulated by the computationally selected miRNAs. In particular, two different approaches were adopted to evaluate the involvement of selected miRNAs in the pathogenesis of CRC and tumors. First, a pathway research was performed by searching all the molecular pathways known to be involved in CRC development as described in literature and according to the indications of The Cancer Genome Atlas Network in reference to CRC.

A second analysis was performed by using the selected miRNAs as search terms in order to unveil all the pathways where these miRNAs modulate the expression levels of genes. Subsequently, the main pathways involved in tumor development and in CRC were selected, including colorectal cancer pathway, pathway in cancer, Ras signaling pathway, etc.

Therefore, such analyses allowed the identification of the most altered pathways associated with the development of CRC as well as the list of genes targeted by miRNAs.

3.4 Patients and sample included in the study

To validate the predictive role of miRNAs dysregulation in CRC, the expression levels of the computationally selected miRNAs were evaluated in a case series of CRC patients and healthy donors. From CRC patients, both liquid biopsy samples and FFPE samples were collected. In detail, both tumor and adjacent normal mucosa of FFPE samples were obtained from each participant.

Overall, 34 CRC patients were enrolled in the study and the tissue expression levels of the selected miRNAs were evaluated in FFPE tumor samples and matched normal colon mucosa by using samples deposited in the biobank of the University of Catania (samples kindly provided by Professor Antonio Di Cataldo

and Professor Gaetano Magro). More in detail, from each patient, 10 FFPE sections of tumor tissue and 10 FFPE sections of adjacent normal mucosa of 5-8 μm were obtained.

A different cohort of patients was investigated for the analysis of the circulating levels. In particular, 14 patients with a confirmed diagnosis of CRC were recruited within the two clinical trials promoted by the University of Catania and aimed at evaluating the beneficial effects of LGG probiotics administration in cancer patients treated with chemotherapy and abdominal radiation therapy. For all these patients, liquid biopsy samples were collected. In addition, liquid biopsy samples were also obtained from 10 healthy donors and used as normal controls. In particular, two peripheral blood draws were collected from each patient in order to separate serum (tube with separating gel) and plasma, buffy coat and red cells (tube with K3 EDTA) after centrifugation at 2,000 g for 10 minutes at room temperature. The aliquots thus obtained were then stored at -80°C until their use.

3.5 RNA Extraction and microRNA Reverse Transcription

The extraction of the miRNAs from FFPE and serum samples was performed using two different protocols.

The miRNeasy FFPE kit (Qiagen - Cat. No. 217504) was used for the extraction of miRNAs from FFPPE samples. In particular, total RNA, including miRNAs, was extracted from four tissue sections of 5-8 μm for both tumor samples and adjacent normal mucosa as per protocol.

The extraction of circulating miRNAs from liquid biopsy samples was performed using the miRNeasy Serum/Plasma kit (Cat. No. 217184, Qiagen, Hilden, Germany). In particular, circulating miRNAs were extracted from 200 μL of serum using the manufacturer's instructions but modifying some steps of the protocol. More in detail, each serum sample from CRC patients and healthy controls was centrifuged at 2000g x 10 min at room temperature. This step allowed us to obtain from each sample an aliquot of serum without any debris and protein aggregates that could interfere with the extraction reaction. Subsequently, the exogenous synthetic UniSp4 spike-in control (Cat. No. 339390, Qiagen, Hilden, Germany) was added to 200 μL of serum sample and

then the circulating miRNAs were extracted using molecular grade reagents (ethanol and chloroform). The UniSp4 spike-in control was used to normalize the absolute quantification levels of extracted circulating miRNAs.
Finally, 4 μL of extracted miRNAs were reverse transcribed into cDNA using the miRCURY LNA RT Kit (Cat. No. 339340, Qiagen, Hilden, Germany).

3.6 Absolute quantification of miRNAs expression levels

For the evaluation of miRNA expression levels in both FFPE and liquid biopsy samples, a novel and high-sensitive custom Qiagen-Bio-Rad ddPCR protocol was adopted. For the precise analysis of miRNA expression levels, Qiagen LNA primers specific to the four selected miRNAs were adopted. In addition, specific primers were also used for the analysis of the abundance of Unisp4 spike-in control (miRCURY LNA miRNA PCR Assays x200, Qiagen - Cat. No. 339306) and endogenous U6 snRNA used as control small RNAs for the quantification of miRNA expression levels in liquid biopsy and FFPE samples, respectively. Noteworthy, the use of ddPCR guarantees more reliable results compared to other conventional techniques like RT-qPCR as it can be used for the analysis of low-quality samples like FFPE or liquid biopsy samples where the nucleic acids are strongly degraded or a low amount of target is present.
In particular, the ddPCR assay is based on an emulsion between water and oil solutions which allows the nano-partition of the sample into thousands of nano-droplets where single reactions were run. In this way, specific primers, probes or EvaGreen fluorescent dye, Taq polymerase, amplification buffer and the nucleic acids target will be theoretically contained within each droplet. More in detail, in our experiment, for each sample analyzed, a reaction mix was prepared using 11 μL of 2x QX200 ddPCR EvaGreen® Supermix (Cat. No. 1864034 - Bio-Rad, Hercules, California, USA), 1.1 μL of miRNA-specific primer miRCURY LNA miRNA PCR Assay (Cat. No. 339306 Qiagen, Hilden, Germany) 6.9 μL of RNase and DNase free-water, and 3 μL of cDNA for a total reaction volume of 22 μL.
Subsequently, 20 μL of reaction mix was used to generate about 20,000 droplets with the QX200 droplet generator (Bio-Rad, Hercules, California, USA). After the generation of droplets, each sample was transferred into a 96-well plate,

sealed and amplified in a C1000 Touch Thermal Cycler (Bio-Rad, Hercules, California, USA). The amplification protocol consists in a first step of polymerase activation for 5 minutes at 95 °C followed by 40 amplification cycles composed by a denaturation phase at 95 °C for 30 seconds and an annealing phase at 56 °C for 1 minute; subsequently the amplification protocol consists of signal stabilization phase at 4 °C for 5 min and 90 °C for 5 min followed by an infinite hold at 4 °C. The ramp rate between the different steps is 1,6 ° C/s. After amplification, negative and positive droplets were read in the QX200 Droplet Reader (Bio-Rad, Hercules, California, USA). All experiments were performed in triplicate (Figure 8).

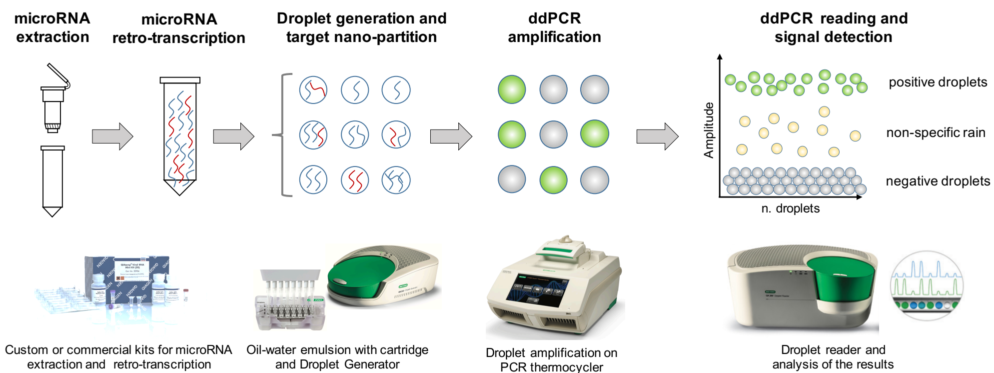

Figure 8. ddPCR workflow for the analysis of miRNAs.

3.7 Bioinformatics analyses

Further bioinformatics analyses were performed after the validation of the diagnostic potential of the four selected miRNAs, hsa-miR-21-5p, hsa-miR-375, hsa-miR-497-5p, hsa-miR-503-5p, in order to assess the correlation between these miRNAs and clinical-pathological features of patients affected by CRC.

The bioinformatics software miRTargetLink Human was used to identify the genes targeted by the miRNAs investigated through ddPCR. The use of this software made it possible to identify the genes targeted by the four miRNAs, through the analysis of the miRNA-mRNA interaction data deposited on different databases, among which miRBase, KEGG Pathway, TargetScan, etc. In particular, two analyses were conducted:

- Identification of genes strongly targeted by the selected miRNAs
- Identification of genes that weakly interact with the selected miRNAs

The genes strongly modulated by miRNAs as predicted by miRTargetLink Human were additionally analyzed with GO enrichment tools, like STRING (Search Tool Retrieval of Interacting Genes/Proteins) and GO Panther software to assess the functional roles of the genes targeted by miRNAs in CRC.
Finally, other two tools were used to analyze the clinical data contained in TCGA COAD and TCGA READ databases. Particularly, GEPIA (Gene Expression Profiling Interactive Analysis) was used to evaluate the dysregulation of the genes targeted by the four validated miRNAs while OncoLnc was used to evaluate the prognostic significance of the selected miRNAs in the prediction of the overall survival of COAD and READ patients.

3.8 Statistical analyses

All the computational analyses conducted and the bioinformatics tools adopted already perform data normalization and statistical analyses to obtain only statistically significant results. In particular, the GEO2R software analyzes only already normalized data obtained through different microarray platforms. Thus, no further normalization was required for the data obtained from all datasets included in the study. The same software was also used to perform the differential analyses of the expression levels between tumor and normal samples and colon mucosa before and after the administration of probiotics.
As regards the statistical analyses related to the targeted genes and pathways, the software mirDIP and DIANA-mirPath software were used.
The miRNAs expression levels data observed in liquid biopsy samples through ddPCR were normalized according to the expression levels of the exogenous control used, uniSp4, while the expression levels obtained in FFPE samples were normalized using the endogenous control snRNA U6.
The raw data obtained through ddPCR were analyzed through the QuantaSoft software, provided by Bio-Rad, which permits the absolute quantification of miRNA expression in serum and FFPE tissue samples. Additionally, the statistical analyses conducted on these data include the Kolmogorov-Smirnov normality test, used to evaluate the distribution of expression levels of hsa-miR-21-5p, hsa-miR-497-5p, hsa-miR-503-5p and hsa-miR-375; the Wilcoxon test was used to establish the statistical differences between tumor FFPE samples

and adjacent normal mucosa; the Mann-Whitney test was used in order to conduct the statistical analysis of miRNAs expression levels in liquid biopsy samples. The specificity and sensitivity of the analyzed miRNAs were assessed by calculating the Receiver Operating Characteristic (ROC) curves. The statistical analyses were conducted using GraphPad Prism v.8.

4. RESULTS

4.1 Computational identification of miRNAs associated with colorectal cancer development

By using the search terms described in the methods section, it was possible to identify 113 different miRNAs expression datasets related to CRC.

By applying the inclusion and exclusion criteria, it was possible to select 13 different datasets. Table 1 contains the datasets selected for the subsequent differential analyses (Table 1).

Table 1. Main features of the selected datasets.

Series Accession	n. normal	n. cancer	Samples	Platform	Author ref.	Total Samples
GSE18392	29	116	Normal colon tissues and colon tumor tissues	Illumina Human v1 MicroRNA expression beadchip	Sarver AL et al, 2009. BMC Cancer. 9: 401.	145
GSE108153	21	21	Paired tumour tissues and adjacent normal tissues	Agilent-046064 Unrestricted Human miRNA V19.0 Microarray	Zeng Z et al, 2017 (NO REF)	42
GSE30454	20	54	Normal colonic mucosa and RNA from formalin-fixed paraffin-embedded tissue blocks from 4 different CRC groups	Illumina Human v2 MicroRNA expression beadchip	Balaguer F et al, 2011. Clin Cancer Res. 17: 6239-49.	74
GSE35834	23	31	Normal adjacent mucosa, primitive colorectal cancer and liver metastasis tissues	[miRNA-1_0] Affymetrix miRNA Array	Pizzini S et al, 2013. BMC Genomics. 14: 589.	78
GSE38389	71	69	Tumor biopsies and corresponding matched mucosa samples	Exiqon miRCURY LNA microRNA array v.9.2 Extended Version	Gaedcke J et al, 2012. Clin Cancer Res. 18: 4919-30.	140
GSE41012	15	20	CR distant normal mucosa and different stages of CR primary tumor	Exiqon miRCURY LNA microRNA Array, v. 9.2, all organisms	Li X, 2015. (NO REF)	35
GSE41655	15	33	Human colorectal tissues, including normal mucosa, adenoma and adenocarcinoma.	Agilent-021827 Human miRNA Microarray [miRNA_107_Sep09_2_105]	Shi X, Zhang Y, Cao B, et al, 2015 (NO REF)	107
GSE49246	40	40	Adjacent normal tissues and stage II colon tumor tissues	Sun Yat-Sen University Cancer Center Human microRNA array	Zhang JX et al, 2013. Lancet Oncol. 14: 1295-306.	80
GSE68204	8	37	Normal rectal biopsies and tumor rectal biopsies	Agilent-021827 Human miRNA Microarray (V3) (miRBase release 12.0 miRNA ID version)	Millino C et al, 2017. J Cell Physiol. 232: 426-435.	125
GSE83924	20	20	Fresh frozen tissue samples from tubular and tubulovillous adenoma and colorectal adenocarcinoma	[miRNA-3] Affymetrix Multispecies miRNA-3 Array	Nagy ZB, Wichmann B, Molnár B, 2016. (NO REF)	60

For all the selected datasets, the differential analyses between the expression levels of miRNAs observed in colorectal cancer samples compared to those observed in normal controls were performed. Overall, the 13 datasets selected contained the expression levels of miRNAs of a total of 441 tumor samples and 262 normal controls. Of note, only datasets contained tissue samples were selected, thus excluding datasets related to liquid biopsy samples that could introduce analytical biases in the analysis of miRNAs due to miRNA contamination from other tissues or organs.

By performing differential analyses through GEO2R and by merging the lists of dysregulated miRNAs obtained from each dataset, it was possible to identify a total of 39 dysregulated miRNAs in CRC patients. Among these, only the miRNAs with concordant expression levels among the datasets in which they were expressed were selected.

After this further selection, a panel of 20 strongly dysregulated miRNAs in CRC was obtained by considering only the top 20 dysregulated miRNAs among the datasets.

Specifically, ten of these miRNAs were significantly and strongly up-regulated while ten miRNAs were down-regulated as shown in Figure 9.

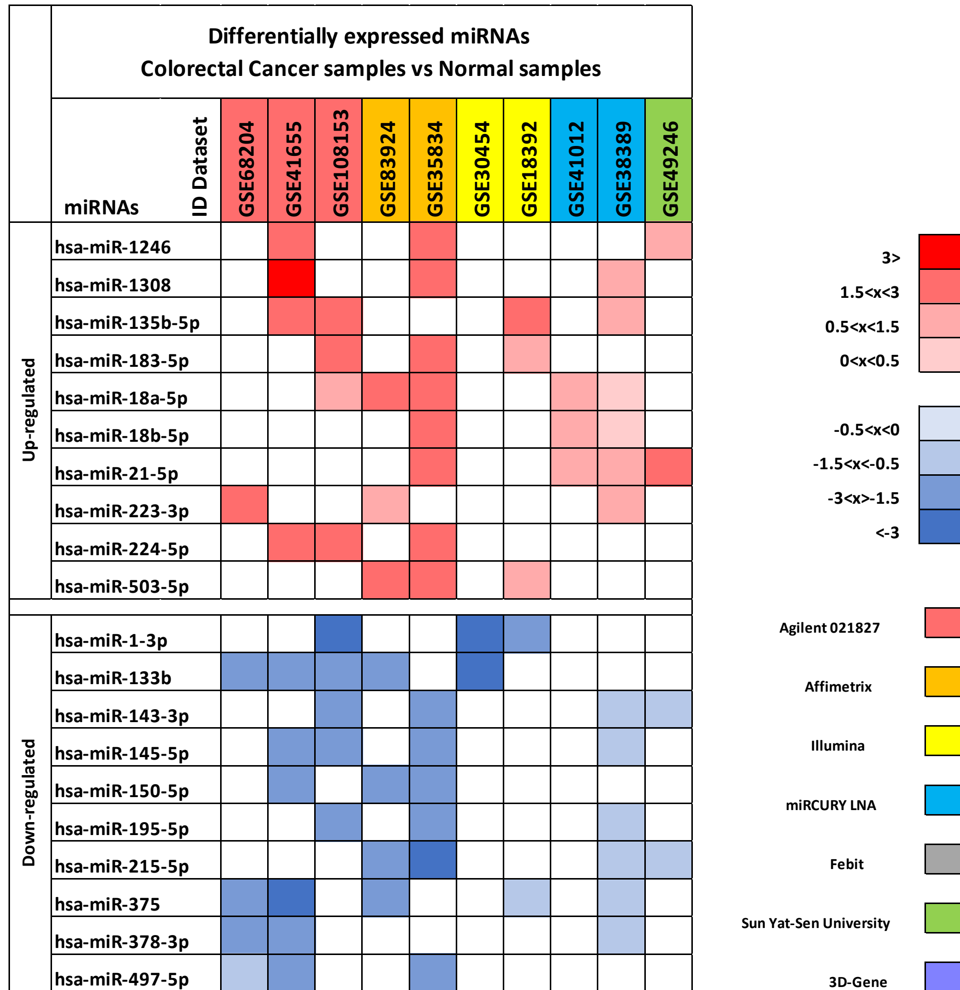

Figure 9. List of dysregulated miRNAs in CRC.

Notably, among these miRNAs there are some miRNAs known to be involved in the alteration of several signal transduction pathways related to tumor development, acting as oncomiR or anti-oncomiR. For example, hsa-miR-21-5p and hsa-miR-375 were, respectively up- and down-regulated in the majority of the datasets analyzed. Some studies have already described the implication of these and other miRNAs, like hsa-miR-18a-5p and hsa-miR-497-5p in the

development of CRC [Campayo M. et al., 2018; Zhou L. et al., 2018; Zou G. et al., 2019]. Other miRNAs emerged through this analysis. Among these miRNAs, hsa-miR-1308 was excluded from further computational analyses due to its nature of tRNA-fragment and not a real miRNA.

To confirm the validity of such analysis performed on heterogenous microarray miRNA expression dataset, further investigations were performed on the miRNA expression data contained in The Cancer Genome Atlas PANCAN database.

By performing differential analyses of the expression levels of miRNAs between CRC samples and normal solid tissue it was obtained a list of 225 miRNAs significantly dysregulated in CRC, of which 145 were down-regulated and 80 were up-regulated (Log2FC ± 1.5, $p<0.01$, Appendix A – Supplementary Table 1).

Finally, by merging the list of miRNAs obtained from the analysis of GEO DataSets microarray platforms with the list of miRNAs obtained from the analysis of the TCGA PANCAN database it was possible to note that five up-regulated miRNAs and nine down-regulated miRNAs were shared between the two lists suggesting how both computational analyses were rigorous in selecting miRNAs effectively involved in the development of CRC. More in detail, the five up-regulated miRNAs were hsa-miR-21-5p, hsa-miR-503-5p, hsa-miR-183-5p, hsa-miR-223-3p and hsa-miR-224-5p. As regards the nine common down-regulated miRNAs there were hsa-miR-133b, hsa-miR-143-3p, hsa-miR-145-5p, hsa-miR-150-5p, hsa-miR-195-5p, hsa-miR-215-5p, hsa-miR-375-3p, hsa-miR-378a-3p and hsa-miR-497-5p, however, the miRNAs hsa-miR-143-3p, hsa-miR-195-5p and hsa-miR-215-5p showed discordant expression levels as they were down-regulated in the GSE datasets and up-regulated in the TCGA PANCAN database, therefore, these three miRNAs cannot be considered potential biomarkers for the diagnosis of CRC.

After the identification of these 20 miRNAs obtained from GEO DataSets and partially confirmed by the analysis of TCGA PANCAN database, further bioinformatics analyses were performed in order to clarify the functional roles of the 20 dysregulated miRNAs.

First, COSMIC database was consulted in order to identify the ten most altered genes in CRC. This preliminary analysis made possible to select the following genes: APC (51%), TP53 (46%), KRAS (34%), FAT4 (23%), LRP1B (20%), TGFBR2 (17%), PIK3CA (15%), KMT2C (13%), ZFHX3 (12%) e BRAF (10%) (Figure 10):

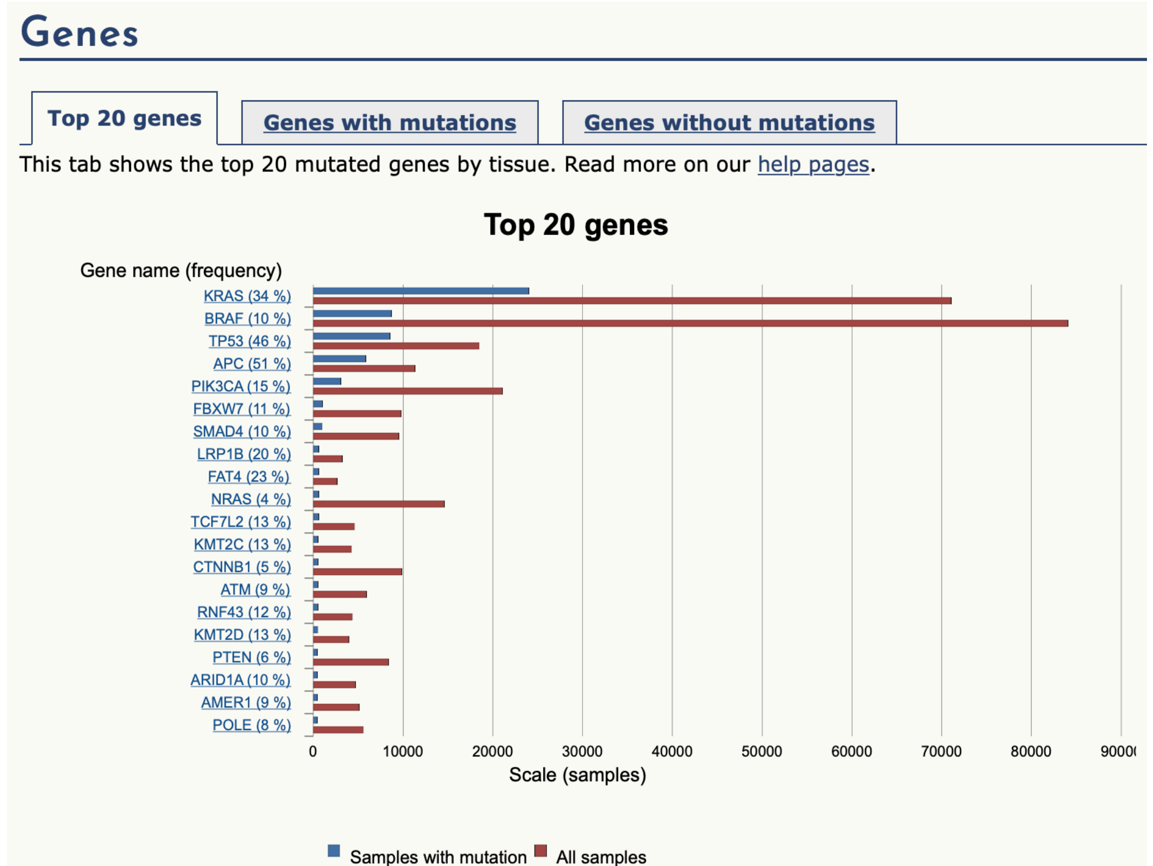

Figure 10. Main mutated and altered genes in CRC.

After the selection of these genes involved in CRC, the mirDIP software was used to evaluate the interaction levels between the 19 selected miRNAs (hsa-miR-1308 excluded as not a miRNA but a tRNA fragment) and the top altered genes in CRC resulted from COSMIC analysis. In this manner it was possible to highlight "medium" to "very high" interaction levels between the selected miRNAs and the altered genes (Figure 11).

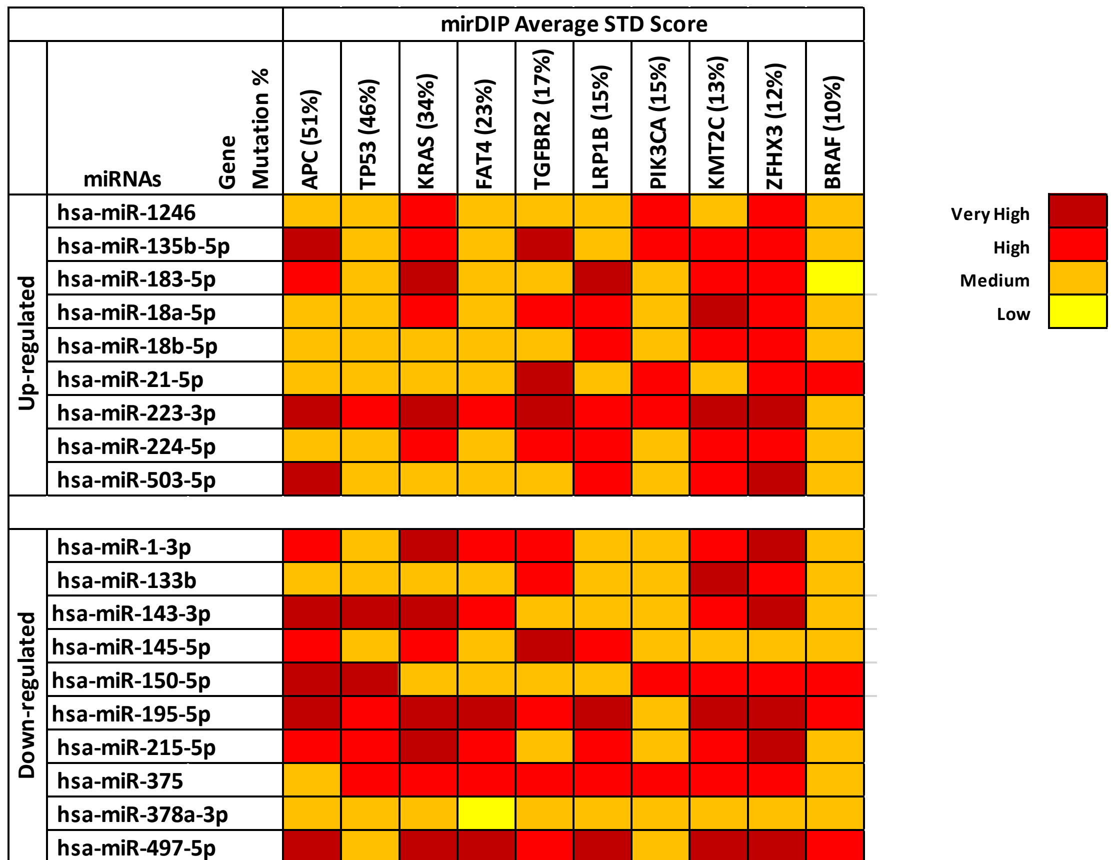

Figure 11. miRNA-mRNA interaction levels according to mirDIP data between the computationally selected miRNAs and main altered genes in CRC.

Taking into account the genes, the most targeted by the selected miRNAs were ZFHX3 and KMT2C; while, as regards miRNAs the higher interaction levels were showed by hsa-miR-223-3p and hsa-miR-183-5p (up-regulated miRNAs) and hsa-miR-195-5p, hsa-miR-497-5p, hsa-miR-150-5p and hsa-miR-375 (down-regulated miRNAs) (Figure 11).

Finally, the functional roles of the selected miRNAs were further evaluated by performing DIANA-mirPath pathway prediction analysis.

This further computational investigation performed on the 19 selected highly-modulated miRNAs in CRC was conducted to establish the genes and pathways modulated by the selected miRNAs. As described in the methods section, two different approaches were performed. In the first approach, the pathway prediction analysis was performed by searching the most altered pathways in CRC.

First, the seven pathways most altered in CRC according to TCGA guidelines were investigated through DIANA-mirPath. The analysis revealed as all the selected miRNAs were able to modulate the Wnt signaling pathway (hsa04310), RAS signaling pathways (hsa04041), MAPK signaling pathways (hsa04010), PI3K-AKT signaling pathway (hsa04151), TGF-β signaling pathways (hsa04350), p53 signaling pathway (hsa04115) and the mismatch repair pathway (hsa03430).

Among all these pathways, the most targeted and altered were the MAPK signaling pathways (hsa04010) and the PI3K-AKT signaling pathway (hsa04151). Focusing the attention on genes, hsa-miR-183-5p, hsa-miR-21-5p, both up-regulated and hsa-miR-195-5p, hsa-miR-497-5p, both down-regulated, were able to target a total of 214 different genes within these pathways. Some of these genes were TP53, APC, genes of the WNT family (WNT3A, WNT5A and WNT9A), of the MAPK family (MAPK1, MAPK8 and MAPK9), and other key oncogenes like MYC and VEGFA. Therefore, these data strongly support the effective pathogenetic role of the miRNAs computationally identified as able to alter key genes involved in CRC development (Table 2).

Table 2. Involvement of the computationally selected miRNAs in different TCGA Colorectal Cancer pathways according to DIANA-mirPath analysis v.3.

miRNAs	p Value	Targeted genes	Main genes
Wnt signaling pathway (hsa04310)			
hsa-miR-1246	9.08E-08	4	DVL3, TP53, PPP3CA, NFATC3
hsa-miR-135b-5p	7.52E-04	2	APC, MYC
hsa-miR-183-5p	1.12E-26	12	TCF7L2, ROCK2, TP53, CCND1, SMAD4, CSNK1A1, MYC, CSNK2A1, RAC1, PSEN1, NFATC3, PLCB4
hsa-miR-18a-5p	1.29E-21	10	DAAM2, CCND2, SMAD3, JUN, CCND1, AXIN2, CSNK1A1, CSNK2A1, FOSL1, NFATC3
hsa-miR-18b-5p	9.61E-06	3	CCND2, CCND1, CSNK1A1
hsa-miR-21-5p	6.62E-32	14	BTRC, APC, WNT5A, CCND2, ROCK2, AXIN1, CCND1, CTNNB1, CSNK1A1, MYC, CSNK2A1, PRICKLE2, BAMBI, TBL1XR1
hsa-miR-223-3p	\	\	\
hsa-miR-224-5p	6.77E-10	5	GSK3B, LRP6, SMAD4, MAPK8, TBL1XR1
hsa-miR-503-5p	9.61E-06	3	CCND2, CSNK2A1, CCND3
hsa-miR-1-3p	9.57E-17	8	CSNK2A2, CAMK2G, CTBP1, CTBP2, PLCB3, CCND1, SFRP1, DKK1
hsa-miR-133b	3.88E-02	1	EP300

hsa-miR-143-3p	9.08E-08	4	CSNK2A2, SENP2, FZD2, EP300
hsa-miR-145-5p	1.12E-26	12	FZD7, GSK3B, PRKCA, WNT5A, WNT5B, PPP3CA, CCND1, CTNNB1, MYC, RAC1, WNT11, PRKACB
hsa-miR-150-5p	9.61E-06	3	SMAD3, TP53, EP300
hsa-miR-195-5p	2.00E-48	20	CTNNBIP1, DVL3, BTRC, CCND2, CUL1, MAPK9, CCND1, CTNNB1, MAPK8, CSNK1A1, MYC, PRKACA, EP300, WNT3A, BAMBI, NFATC3, CREBBP, TBL1XR1, WNT9A, CCND3
hsa-miR-215-5p	3.88E-02	1	FZD5
hsa-miR-375	3.74E-19	9	PRKCA, VANGL1, CHD8, RHOA, FZD8, FZD4, JUN, MYC, PRKX
hsa-miR-378a-3p	1.12E-26	12	VANGL1, CCND2, ROCK2, PPP3R1, JUN, CCND1, DVL1, MYC, EP300, RBX1, NFATC3, TBL1XR1
hsa-miR-497-5p	1.41E-34	15	DVL3, BTRC, WNT5A, CCND2, ROCK2, SMAD3, NLK, CCND1, MAPK8, CSNK1A1, MYC, CSNK2A1, TBL1XR1, WNT9A, CCND3
RAS signaling pathway (hsa04041)			
hsa-miR-1246	1.00E-07	5	PIK3CB, CALM2, PTPN11, RASGRP3, RAB5B
hsa-miR-135b-5p	\	\	\
hsa-miR-183-5p	2.56E-32	17	NRAS, STK4, IGF1R, GNB1, KRAS, PAK1, PTPN11, RAC1, FGF2, PDGFC, RALGDS, MAPK1, ABL2, GNG5, RAB5B, EFNA1, RASSF5
hsa-miR-18a-5p	3.48E-15	9	STK4, PAK2, ETS2, GNG12, RAB5A, KIT, ARF6, RAB5C, GRIN2A
hsa-miR-18b-5p	1.00E-07	5	STK4, GNG12, ARF6, RAB5C, GRIN2A
hsa-miR-21-5p	7.62E-54	26	NFKB1, SOS2, NRAS, PAK2, GNG12, RALA, PLD1, TIAM1, IGF1R, EGFR, FASLG, MLLT4, AKT2, RASGRP1, REL, PIK3R1, TBK1, EPHA2, NF1, GAB1, PDGFD, RASGRP3, VEGFA, MAPK1, GNB4, TEK
hsa-miR-223-3p	4.84E-06	4	IGF1R, PIK3CD, RASGRP1, VEGFA
hsa-miR-224-5p	1.85E-23	13	PDGFRA, NRAS, CALM3, CALM1, PAK2, ETS1, EFNA3, IGF1R, EGFR, PIK3R3, MAPK8, CDC42, PDGFRB
hsa-miR-503-5p	3.48E-15	9	IGF1R, RRAS2, IKBKB, RASA1, FGF2, AKT3, HGF, VEGFA, ABL2
hsa-miR-1-3p	1.26E-25	14	MET, RIN1, SOS2, CALM3, CALM1, EGFR, CALM2, GNB2, AKT1, CDC42, EXOC2, PIK3CA, FGFR2, RAP1B
hsa-miR-133b	1.00E-07	5	MET, CALM1, IGF1R, EGFR, FGFR1
hsa-miR-143-3p	3.48E-15	9	CALM1, KRAS, AKT1, REL, PTPN11, HRAS, MAPK1, ABL2, RASSF5
hsa-miR-145-5p	7.99E-28	15	PRKCA, GNG11, NRAS, ANGPT2, EGFR, PAK1, RASA1, AKT1, RAC1, PAK4, ARF6, VEGFA, ABL2, PDGFRB, PRKACB
hsa-miR-150-5p	4.84E-06	4	MET, GNG11, VEGFA, FGFR1
hsa-miR-195-5p	4.88E-44	22	CALM1, CHUK, IGF1R, RRAS2, IKBKB, RASA1, MAPK9, GNB2, PRKACA, FGF2, CDC42, NF1, AKT3, PDGFC, PIK3CA, HGF, VEGFA, GRB2, PDGFA, EFNA1
hsa-miR-215-5p	5.33E-03	2	GAB1, RGL1
hsa-miR-375	1.85E-23	13	PRKCA, PDGFRA, FGFR3, CALM3, RAF1, IGF1R, RHOA, REL, PRKX, CDC42, PDGFC, ARF6, ABL1

hsa-miR-378a-3p	1.86E-04	3	CALM1, GNG12, RAB5B
hsa-miR-497-5p	4.88E-44	22	RIN1, SOS2, CALM1, PAK2, PIK3R2, CHUK, IGF1R, CALM2, IKBKB, AKT2, GNB2, MAPK8, FGF2, CDC42, AKT3, PIK3CA, MAP2K1, HGF, VEGFA, MAPK1, ABL2, GRB2
MAPK signaling pathway (hsa04010)			
hsa-miR-1246	6.77E-09	6	TAOK1, TP53, PPP3CA, MAP3K2, RASGRP3, NFATC3
hsa-miR-135b-5p	8.31E-03	2	CHUK, MYC
hsa-miR-183-5p	5.84E-35	19	FOS, HSPA1A, ATF2, NRAS, CRKL, STK4, ELK4, MAP3K13, KRAS, PAK1, TP53, RAPGEF2, MYC, RAC1, FGF2, MAP3K2, HSPA1B, NFATC3, MAPK1
hsa-miR-18a-5p	1.45E-30	17	MAPK8IP2, STK4, ELK4, PAK2, CACNB4, GNG12, MAP4K4, MAP3K1, TAOK1, JUN, HSPA8, DUSP3, STMN1, MAPK3K2, NFATC3, DUSP1, TGFBR2
hsa-miR-18b-5p	3.13E-07	5	STK4, ELK4, GNG12, HSPA8, STMN2
hsa-miR-21-5p	3.02E-68	33	NFKB1, SOS2, MAP4K2, ATF2, NRAS, CRKL, IL1B, PAK2, MAP2K7, GNG12, MAP2K3, EGFR, MAP3K4, TGFB1, FASLG, TAOK1, AKT2, DUSP10, RASGRP1, MYC, HSPA8, NF1, FAS, RPS6KA3, STMN1, MAP3K2, RASGRP3, MEF2C, MKNK2, DUSP16, MAPK1, TGFBR2, DAXX
hsa-miR-223-3p	1.20E-05	4	DUSP10, RASGRP1, STMN1, MEF2C
hsa-miR-224-5p	3.92E-22	13	PDGFRA, NRAS, PAK2, EGFR, TAB2, TAOK1, MAPK8, ZAK, CDC42, STMN1, PDGFRB, TGFB3, NR4A1
hsa-miR-503-5p	2.81E-14	9	DUSP2, CRKL, RRAS2, IKBKB, RASA1, FGF2, HSPA8, AKT3, CACNA2D1
hsa-miR-1-3p	3.41E-24	14	HSAPA1A, SOS2, MAP4K2, EGFR, BDFN, RAPGEF2, AKT1, FLNB, FLNA, CDC42, CACNA2D1, FGFR2, HSPA1B, RAP1B
hsa-miR-133b	8.31E-03	2	EGFR, FGFR1
hsa-miR-143-3p	3.53E-16	10	ELK4, MAPK7, TAB2, KRAS, AKT1, CACNA1E, HRAS, MKNK2, DUSP16, MAPK1
hsa-miR-145-5p	9.63E-42	22	PRKCA, NRAS, ELK4, MAPK14, MAP4K4, EGFR, TGFB1, TAOK, PAK1, MAP2K6, PPP3CA, RASA1, AKT1, MYC, RAC1, STK3, HSPA8, TGFB2, MAP3K5, PDGFRB, PRKACB, TGFB3
hsa-miR-150-5p	1.20E-05	4	PTPRR, TP53, FGFR1, DUSP16
hsa-miR-195-5p	4.18E-51	26	GNA12, ATF2, DUSP2, CRKL, CRK, MAPK14, CHUK, RRAS2, TAOK1, RPS6KA5, IKBKB, RASA1, MAPK9, MAPK8, MYC, PRKACA, FGF2, CDC42, NF1, RPS6KA3, AKT3, CACNA2D1, MKNK2, NFATC3, GRB2, PDGFA
hsa-miR-215-5p	1.20E-05	4	MAP3K13, TAOK1, MKNK2, HSPA1B
hsa-miR-375	9.47E-33	18	PRKCA, PDGFRA, DUSP6, RAF1, GADD45A, MAP3K1, TAOK1, CASP3, JUN, MYC, FLNB, CDC42, HSPA8, MAP3K8, MAP2K4, SRF
hsa-miR-378a-3p	5.84E-35	19	HSPA1A, CRKL, GNG12, PPP3R1, JUN, RAPGEF2, MYC, FLNA, MAPT, HSPA8, NF1, DUSP7, STMN1, HSPA1B, NFATC3, MAPK1, GRB2, RAP1B, ELK1

hsa-miR-497-5p	3.39E-37	20	SOS2, ATF2, CDC25B, PAK2, DUSP6, CHUK, IKBKB, AKT2, NLK, MAPK8, MYC, FGF2, CDC42, HSPA8, RPS6KA3, AKT3, MAP2K1, CACNA2D1, MAPK1, GRB2
PI3K-AKT signaling pathway (hsa04151)			
hsa-miR-1246	7.91E-09	7	PIK3CB, PPP2CA, BCL2, CDK6, TP53, CCNE1, PPP2R1B
hsa-miR-135b-5p	2.33E-07	6	THBS1, YWHAG, CHUK, MYC, COL4A3, PHLPP1
hsa-miR-183-5p	8.89E-55	31	PRLR, ITGB1, ITGB8, ATF2, NRAS, ITGA8, ATF6B, PPP2CA, YWHAG, MCL1, PCK2, HSP90AA1, ITGA5, IGF1R, GNB1, PPP2RD, PPP2R5C, KRAS, TP53, CCND1, CCNE2, MYC, RAC1, FGF2, PDGFC, EIF4E2, CREB3L2, PTEN, MAPK1, GNG5, EFNA1
hsa-miR-18a-5p	1.03E-18	13	IL2RG, COL27A1, CDKN1B, KRAS, RHEB, FGF11, PIK3R3, EIF4B, FOXO3, CDKN1A, SGK3, COL4A1, EFNA1
hsa-miR-18b-5p	2.34E-10	8	PPP2R3A, CCND2, GNG12, CCND1, ITGA2, CDKN1A, PPP2R1B, MDM2
hsa-miR-21-5p	2.12E-77	41	PRLR, NFKB1, SOS2, LAMB1, ATF2, NRAS, PRKAA2, THBS1, CCND2, GNG12, BCL2, IGF1R, EGFR, TRL4, CDK6, FASLG, AKT2, PTK2, BRAC1, CCND1, F2R, MYC, HSP90AB1, PIK3R1, HSP90AB1, PIK3R1, HSP90B1, EPHA2, LAMC1, COL6A3, PDGFD, FOXO3, VEGFA, PTEN, SGK3, MAPK1, PPP2R1B, COL5A2, GNB4, TEK, MDM2, COL4A1, RXRA
hsa-miR-223-3p	7.91E-09	7	BCL2, IGF1R, DDIT4, PIK3CD, HSP90B1, MTOR, VEGFA
hsa-miR-224-5p	5.85E-28	18	GSK3B, PDGFRA, NRAS, MCL1, PPP2R5D, EFNA3, IGF1R, EGFR, PIK3R3, PRKAA1, EIF4E2, FN1, PKN2, TNC, CDKN1A, PDGFRB, COL4A1, NR4A1
hsa-miR-503-5p	2.53E-22	15	RBL2, MCL1, CCND2, BCL2, IGF1R, IKBKB, COL6A1, CCNE2, FGF2, LAMC1, AKT3, LAMC2, HGF, VEGFA, CCND3
hsa-miR-1-3p	3.59E-46	27	MET, SOS2, CDK4, COL6A5, THBS1, THBS2, ITGA3, BCL2, EGFR, TLR4, YWHAQ, PPP2R5A, BRCA1, GNB2, CCND1, EIF4E, AKT1, PPP2R2A, COL1A1, HSP90B1, PIK3CA, FN1, FGFR2, ITGA6, SGK3, IL6, PPP2R1B
hsa-miR-133b	2.33E-07	6	MET, MCL1, EIF4EBP1, IGF1R, EGFR, FGFR1
hsa-miR-143-3p	5.85E-28	18	CDK4, THBS1, BCL2, YWHAB, KRAS, CDK6, IFNAR2, YWHAQ, CRTC2, AKT1, PPP2R2A, RPTOR, COL5A1, COL1A1, HRAS, CREB3L2, MAPK1, MDM2
hsa-miR-145-5p	1.20E-63	35	GSK3B, PRKCA, ITGB1, ITGB8, CDK4, GNG11, NRAS, THBS1, ANGPT2, ITGA5, ITGA3, EGFR, COL3A1, COL6A1, PTK2, ITGAV, ITGA11, DDIT4, CCND1, EIF4E, AKT1, MYC, COL5A1, COL1A1, HSP90AB1, RAC1, COL1A2, FN1, TNC, CDKN1A, VEGFA, COL5A2, SPP1, MDM2, PDGFRB
hsa-miR-150-5p	6.10E-12	9	MET, MYB, GNG11, CDKN1B, TP53, PPP2CB, EIF4E2, VEGFA, FGFR1

hsa-miR-195-5p	2.12E-77	41	PHLPP2, RBL2, MYB, CDK4, ATF2, IL2RB, YWHAG, MCL1, CCND1, HSP90AA1, CHUK, BCL2, YWHAB, IGF1R, PPP2R5C, CDK6, CREB1, IKBKB, GNB2, CCND1, JAK2, CCNE2, MYC, COL5A1, HSP90AB1, FGF2, PPP2CB, AKT3, PDGFC, CCNE1, PIK3CA, LAMC2, HGF, VEGFA, PPP2R1B, GRB2, SGK1, BCL2L11, CCND3, PDGFA, EFNA1
hsa-miR-215-5p	2.57E-02	2	HSP90AA1, BCL2L11
hsa-miR-375	5.62E-42	25	PRLR, PRKCA, PDGFRA, MYB, ITGB1, COL6A5, FGFR3, PRKAA2, YWHAG, MCL1, HSP90AA1, RAF1, BCL2, YWHAB, IGF1R, RPS6, MYC, HSP90AB1, YWHAZ, PPP2R3C, IRS1, PDGFC, FOXO3, MDM2, BCL2L11
hsa-miR-378a-3p	3.59E-46	27	PPP2R5E, MET, ITGA9, PPP2R3A, YWHAG, CCND2, HSP90AA1, GNG12, BCL2, IGF1R, TLR4, CDK6, COL3A1, IFNAR1, PIK3R3, CCND1, MYC, MLST8, PPP2R1A, PIK3R1, YWHAZ, VEGFA, PTEN, MAPK1, GNG5, GRB2, BCL2L11
hsa-miR-497-5p	1.84E-70	38	SOS2, CDK4, ATF2, PIK3R2, IL2RB, CCND2, HSP90AA1, CHUK, BCL2, IGF1R, RPS6, CDK6, IKBKB, COL6A1, AKT2, GNB2, CCND1, EIF4E, LPAR1, MYC, HSP90B1, FGF2, COL1A2, LAMC1, AKT3, CCNE1, PIK3CA, FN1, MAP2K1, LAMC2, HGF, COL5A3, VEGFA, MAPK1, GRB2, SGK1, MDM2, CCND3
TGF-beta signaling pathway (hsa04350)			
hsa-miR-1246	8.30E-05	2	PPP2CA, PPP2R1B
hsa-miR-135b-5p	8.30E-05	2	THBS1, MYC
hsa-miR-183-5p	2.47E-12	5	PPP2CA, ACVR2B, SMAD4, MYC, MAPK1
hsa-miR-18a-5p	7.60E-18	7	SMAD2, SMAD3, ACVR2A, SMAD7, PPP2R1B, TGFBR2, BMP4
hsa-miR-18b-5p	3.45E-07	3	SMAD2, ACVR2A, PPP2R1B
hsa-miR-21-5p	9.36E-33	12	THBS1, PITX2, TGFB1, ZFYVE16, MYC, SP1, BAMBI, SMAD7, MAPK1, PPP2R1B, TGFBR2, BMPR2
hsa-miR-223-3p	\	\	\
hsa-miR-224-5p	1.05E-09	4	DCN, SMAD4, SMAD5, TGFB3
hsa-miR-503-5p	3.45E-07	3	SMURF2, LTBP1, SMAD7
hsa-miR-1-3p	7.60E-18	7	THBS1, ID4, E2F5, SP1, BMP7, PPP2R1B, BMPR2
hsa-miR-133b	1.30E-02	1	EP300
hsa-miR-143-3p	3.45E-07	3	THBS1, EP300, MAPK1
hsa-miR-145-5p	9.36E-33	12	FST, SMAD2, NODAL, THBS1, ID4, TGFB1, ACVR1, MYC, BMP2, TGFB2, BMP7, TGFB3
hsa-miR-150-5p	2.47E-12	5	SAMD3, BMP2, SP1, EP300, PPP2CB
hsa-miR-195-5p	4.48E-39	14	SMAD2, SMURF2, CUL1, MYC, RBL1, ACVR2A, SP1, EP300, PPP2CB, BAMBI, LTBP1, SMAD7, CREBBP, PPP2R1B
hsa-miR-215-5p	1.30E-02	1	BMP2
hsa-miR-375	2.47E-12	5	CDKN2B, RHOA, MYC, SMURF1, BMPR2
hsa-miR-378a-3p	8.30E-05	2	NODAL, SMAD7
hsa-miR-497-5p	7.60E-18	7	FST, SMAD3, MYC, RBL1, SP1, SMAD7, MAPK1

p53 signaling pathway (hsa04115)			
hsa-miR-1246	6.32E-16	6	BAX, CDK6, TP53, TP53I3, CCNE1, CCNG2
hsa-miR-135b-5p	9.42E-03	1	THBS1
hsa-miR-183-5p	5.54E-25	9	CCNB1, TP53, CCND1, CCNE2, EI24, SESN2, TNFRSF10B, PTEN, GTSE1
hsa-miR-18a-5p	4.33E-05	2	CCND1, RRM2
hsa-miR-18b-5p	4.66E-13	5	CCND2, CCND1, SHISA5, CDKN1A, MDM2
hsa-miR-21-5p	6.78E-38	13	CCNG1, THBS1, CCND2, CDK6, APAF1, CCND1, SESN1, TNFRSF10B, MDM4, FAS, SERPINEB5, PTEN, MDM2
hsa-miR-223-3p	\	\	\
hsa-miR-224-5p	4.66E-13	5	IGFBP3, CDKN1A, RRM2, SERPINE1, CCNG2
hsa-miR-503-5p	4.66E-13	5	ZMAT3, CCND2, RRM2B, CCNE2, CCND3
hsa-miR-1-3p	1.53E-34	9	CDK4, THBS1, BAX, CHEK1, ATM, CCND1, SERPINB5, IGFBP3, ATR
hsa-miR-133b	9.42E-03	1	TNFRSF10B
hsa-miR-143-3p	5.54E-25	9	CDK4, THBS1, PERP, CDK6, APAF1, SESN2, BBC3, SERPINE1, MDM2
hsa-miR-145-5p	2.47E-31	11	CCNG1, CDK4, THBS1, ATM, CCND1, BBC3, CDKN1A, SESN3, SERPINE1, PPM1D, MDM2
hsa-miR-150-5p	2.78E-10	4	BAX, PERP, TP53, MDM4
hsa-miR-195-5p	1.37E-34	12	ZMAT3, CDK4, CCND2, RRM2B, CDK6, CCND1, CCNE1, SHISA5, SESN1, CCNE1, IGFBP3, CCND3
hsa-miR-215-5p			
hsa-miR-375	4.66E-13	5	GADD45A, CASP3, SESN1, IGFBP3, MDM2
hsa-miR-378a-3p	2.47E-31	11	ZMAT3, CCNB1, CCND2, CDK1, CDK6, CHEK1, CCND1, TNFRSF10B, MDM4, PTEN, SERPINE1
hsa-miR-497-5p	1.37E-34	12	ZMAT3, CDK4, CCND2, PERP, RRM2B, CDK6, CCND1, SHISA5, CCNE1, IGFBP3, MDM2, CCND3
Mismatch repair (hsa03430)			
hsa-miR-1246	1.08E-03	1	POLD3
hsa-miR-135b-5p			
hsa-miR-183-5p	1.61E-10	3	PCNA, MSH6, MSH2
hsa-miR-18a-5p	1.08E-03	1	PCNA
hsa-miR-18b-5p			
hsa-miR-21-5p	1.61E-10	3	RFC1, MSH6, MSH2
hsa-miR-223-3p			
hsa-miR-224-5p	1.08E-03	1	RPA1
hsa-miR-503-5p	\	\	\
hsa-miR-1-3p	1.53E-34	9	RPA1, LIG1, POLD1, RFC2, MSH6, MSH2, RFC5, RFC3, EXO1
hsa-miR-133b	\	\	\
hsa-miR-143-3p	\	\	\
hsa-miR-145-5p	\	\	\
hsa-miR-150-5p	\	\	\

hsa-miR-195-5p	1.08E-03	1	RPA2
hsa-miR-215-5p	1.08E-03	1	MSH6
hsa-miR-375	5.36E-07	2	RPA1, EXO1
hsa-miR-378a-3p	5.36E-07	2	MLH3, EXO1
hsa-miR-497-5p	\	\	\

The second pathway prediction analyses performed through DIANA-mirPath revealed a total of 15 molecular pathways related to cancer that were profoundly altered by the selected miRNAs. Three of these pathways are shared between the first and the second approach: PI3K-AKT signaling pathway, TGF- β and p53 signaling pathways.

Through this second approach, a total of 460 univocal genes selectively modulated by the selected miRNAs were identified. All the miRNAs, except for the three miRNAs hsa-miR-215-5p, hsa-miR-1-3p and hsa-miR-503-5p, were able to modulate different molecular pathways. Among all the miRNAs investigated, those with the higher interaction levels with the 15 pathways identified were hsa-miR-145-5p, hsa-miR-195-5p, hsa-miR-378a-3p and hsa-miR-497-5p (Table 3).

Table 3. Interaction between the selected miRNAs and several molecular pathways related to CRC and Cancer according to DIANA-mirPath v.3 analysis.

miRNAs	p Value	Targeted Genes	Gene Target
Proteoglycans in cancer (hsa05205)			
hsa-miR-133b	1.29E-02	6	MET, PPP1CC, IGF1R, EGFR, DDX5, FGFR1
hsa-miR-143-3p	3.95E-03	13	THBS1, KRAS, TFAP4, MMP2, AKT1, IGF2, FZD2, PTPN11, HRAS, MAPK1, PLAU, MDM2, CD44
hsa-miR-145-5p	0.00E+00	40	FZD7, ESR1, ACTB, PRKCA, STAT3, ITGB1, SMAD2, NRAS, THBS1, CAV1, WNT5A, MAPK14, ACTG1, ITGA5, IFGR1, EGFR, WNT5B, CAV2, ERBB3, TGFB1, MSN, PAK1, PTK2, ITGAV, MMP2, CCND1, CTNNB1, AKT1, MYC, MMP9, RAC1, WNT11, TGFB2, NANOG, FN1, CDKN1A, TWIST1, VEGFA, MDM2, PRKACB
hsa-miR-183-5p	2.78E-05	26	BRAF, ACTB, PDCD4, ITGB1, EZR, CBL, NRAS, CAV1, PPP1CC, ROCK2, RDX, IQGAP1, ITGA5, IGF1R, KRAS, PAK1, TP53, PPP1R12A, CCND1, HIF1A, MYC, ITPR1, PTPN11, RAC1, FGF2, MAPK1
hsa-miR-18b-5p	3.24E-02	14	ESR1, BRAF, ACTB, STAT3, SMAD2, MAPK14, CTTN, CCND1, CTNNB1, TIMP3, ITGA2, CDKN1A, MDM2, PPP1CB

hsa-miR-195-5p	2.85E-04	36	ACTB, STAT3, PDCD4, SMAD2, PPP1CC, MAPK14, ACTG1, FRS2, FZD6, IGF1R, RRAS2, MSN, HSPG2, CCND1, CTNNB1, MYC, MMP9, CBLC, PRKACA, DDX5, FGF2, CDC42, AKT3, WNT3A, PIK3CA, CDKN1A, SDC4, VMP1, HGF, VEGFA, ITPR2, PLAU, GRB2, MDM2, WNT9A, CD44
hsa-miR-223-3p	1.79E-02	7	STAT3, IGF1R, MMP2, PIK3CD, MMP9, MTOR, VEGFA
hsa-miR-375	8.27E-05	23	PRKCA, ERBB2, ITGB1, SDC1, IQGAP1, RAF1, IGF1R, RHOA, FZD8, RPS6, ANK2, FZD4, CASP3, HIF1A, MYC, FLBN, PRKX, DDX5, CDC42, TGFB2, PLCE1, CDKN1A, MDM2
hsa-miR-378a-3p	1.48E-03	20	ESR1, ACTB, MET, CBL, SDC1, ARHGEF12, ROCK2, IGF1R, TLR4, MSN, PIK3R3, CCND1, MYC, FLNA, PIK3R1, VEGFA, MAPK1, GRB2, MDM2, ELK1
hsa-miR-497-5p	2.50E-11	43	BRAF, ACTB, PDCD4, SOS2, SMAD2, PTCH1, WNT5A, PIK3R2, PPP1CC, ACTG1, ROCK2, FRS2, FZD6, IQGAP1, IGF1R, RPS6, HSPG2, AKT2, TFAP4, MMP2, CCND1, MYC, MMP9, IGF2, ITPR1, FGF2, CDC42, AKT3, PIK3CA, FN1, CDKN1A, MAP2K1, SDC4, SMO, HGF, MTOR, VEGFA, MAPK1, GRB2, CD63, MDM2, WNT9A, CD44
Hippo signaling pathway (hsa04390)			
hsa-miR-135b-5p	1.77E-02	10	APC, YWHAG, PPP1CC, CCND2, FZD6, BIRC5, MYC, TEAD1, LATS1, LATS2
hsa-miR-145-5p	1.93E-04	25	FZD7, ACTB, GSK3B, SAMD2, NF2, WNT5A, ACTG1, SNAI2, WNT5B, TGFB1, CDH1, CCND1, CTNNB1, MYC, STK3, BMP2, WNT11, TGFB2, BBC3, PARD3, SOX2, BPM7, SERPINE1, CTGF, TGFB3
hsa-miR-183-5p	1.46E-05	18	ACTB, YAP1, PPP2CA, TCF7L2, YWHAG, PPP1CC, MOB1B, PPP2R2D, CDH1, CCND1, SMAD4, MYC, LLGL1, FRMD6, SMAD7, LATS1, LATS2, AJUBA
hsa-miR-195-5p	6.47E-05	28	ACTB, DVL3, YAP1, SMAD2, BTRC, PPP2CA, YWHAG, PPP1CC, CCND2, ACTG1, GLI2, FZD6, BIRC5, YWHAB, AMOT, CDH1, CCND1, CTNNB1, MYC, PPP2R1A, PP2CB, WNT3A, SMAD7, LATS1, PPP2R1B, PARD6B, WNT9A, CCND3
hsa-miR-21-5p	6.53E-03	25	ACTB, TGFBR1, YAP1, BTRC, APC, NF2, WNT5A, DLG1, CCND2, BIRC5, AXIN1, TP53BP2, TGFB1, MPP5, CCND1, CTNNB1, MYC, SAV1, TGFB2, SMAD7, LATS1, SOX2, PPP2R1B, TGFBR2, BMPR2
hsa-miR-224-5p	1.20E-03	18	ACTB, GSK3B, PRKCI, DLG1, YWHAG, PPP1CC, GLI2, FZD6, MPP5, CDH1, CCND1, SMAD4, YWHAZ, LATS1, SERPINE1, PARD6B, BMPR2, TGFB3
hsa-miR-375	1.46E-06	19	YAP1. YWHAG, CCND2, MOB1B, YWHAB, WWC1, AMOT, FZD8, MPP5, FZD4, DLG4, CTNNA1, MYC, YWHAZ, TGFB2, PARD3, PARD6B, BMPR2, CTGF
hsa-miR-378a-3p	8.18E-03	16	ACTB, ID2, YAP1, BTRC, YWHAG, CCND2, MOB1B, MPP5, CCND1, DVL1, CTNNA1, MYC, PPP2R1A, YWHAZ, SERPINE1, DVL2

hsa-miR-497-5p	2.01E-06	27	ACTB, DVL3, YAP1, SMAD2, BTRC, PPP2CA, WNT5A, PPP1CC, CCND2, ACTG1, GLI2, FZD6, SMAD3, BIRC5, AMOT, WWTR1, TP53BP2, CSNK1D, CCND1, MYC, FRMD6, SMAD7, LATS1, LATS2, WNT9A, CCND3, SCRIB
Viral carcinogenesis (hsa05203)			,
hsa-miR-1246	1.71E-05	6	PIK3CB, BAX, CDK6, TP53, KAT2B, CCNE1
hsa-miR-143-3p	2.35E-02	17	CDK4, DLG1, HLA-E, YWHAB, KRAS, CDK6, HIST1H2BD, YWHAQ, EIF2AK2, CCR5, REL, HRAS, EP300, CREB3L2, MPAK1, LYN, MDM2
hsa-miR-150-5p	1.30E-06	9	BAX, PKM, CDKN1B, TP53, EGR2, KIST1H2BG, EP300, HLA-A, HIST1H2BM
hsa-miR-183-5p	1.26E-04	28	CDK4, ATF2, NRAS, ATF6B, YWHAG, HIST1H2BC, PKM, HIST1H4C, HLA-C, KRAS, DDX3X, CHD4, TP53, HNRNPK, EIF2AK2, CCND1, CCNE2, KAT2A, RAC1, RBPJ, GTF2H3, CREB3L2, GTF2A1, MAPK1, HLA-A, HIST1H4J, HIST1H2BO, HIST1H4E
hsa-miR-195-5p	1.31E-05	39	RBL2, STAT3, CDK4, ATF2, CCNA2, YWHAG, CCND2, PKM, HIST1H4C, YWHAB, TRAF5, CDK6, CHD4, CREB1, EIF2AK2, CCND1, CCNE2, UBE3A, PRKACA, DDB1, RBL1, HIST1H2BG, CDC42, EP300, CCNE1, PIK3CA, IL6ST, CDKN1A, TBP, CREBBP, TBPL1, GRB2, MDM2, CCND3, UBR4, VDAC3, HIST1H2BH, HIST1H2BE
hsa-miR-375	2.18E-03	26	CCNA2, GTF2H1, YWHAG, CCND2, HIST1H4C, CDKN2B, YWHAB, RHOA, CREB3, CHD4, EGR3, EIF2AK2, CASP3, JUN, UBE3A, REL, YWHAZ, PRKX, CDC42, RBPJ, BAK1, USP7, CDKN1A, SRF, JAK1, MDM2
hsa-miR-378a-3p	2.01E-02	21	YWHAG, CCND2, PKM, CDK1, CDK6, DDX3X, HIST1H2BD, CHEK1, SND1, JUN, PIK3R3, YWHAZ, HDAC2, EP300, IL6ST, MAPK1, GRB2, TRAF1, MDM2
p53 signaling pathway (hsa04115)			
hsa-miR-1246	4.37E-02	6	BAX, CDK6, TP53, TP53I3, CCNE1, CCNG2
hsa-miR-143-3p	6.25E-03	11	CDK4, THBS1, PERP, CDK6, APAF1, SESN2, TNFRSF10B, BBC3, PTEN, SERPINE1, MDM2
hsa-miR-145-5p	1.50E-02	11	CCNG1, CDK4, THBS1, ATM, CCND1, SESN3, SERPINE1, PPM1D, MDM2
hsa-miR-18a-5p	2.25E-03	13	CCNG1, ZMAT3, CCNB1, CDK4, CCND2, ATM, CCND1, SHISA5, CDKN1A, RRM2, PTEN, PPM1D, MDM2
hsa-miR-378a-3p	2.01E-02	12	ZMAT3, CCNB1, CCND2, CDK1, CDK6, CHEK1, CCND1, TNFRSF10B, MDM4, PTEN, SERPINE1, MDM2
hsa-miR-497-5p	8.21E-04	18	ZMAT3, CCNB1, CDK4, CCND2, PERP, RRM2B, CDK6, CCND1, SHISA5, SESN1, TNFRSF10B, CCNE1, IGFBP3, CDKN1A, SESN3, PIDD1, MDM2, CCND3
Pathways in cancer (hsa05200)			

hsa-miR-145-5p	1.82E-07	41	FZD7, GSK3B, PRKCA, STAT3, ITGB1, CDK4, GNG11, SMAD2, NRAS, WNT5A, ITGA3, IGF1R, EGFR, WNT5B, TGFB1, TPM3, PTK2, ITGAV, CDH1, MMP2, CCND1, CTNNB1, MMP1, AKT1, MYC, MMP9, HSP90AB1, HDAC2, RAC1, WNT11, TGFB2, FN1, CDKN1A, BIRC3, STAT1, VEGFA, MDM2, PDGFRB, PRKACB, TGFB3
hsa-miR-150-5p	1.88E-02	14	MET, GNG11, CXCR4, BAX, SMAD3, CDKN1B, TP53, TCEB2, BMP2, FLT3, EP300, SLC2A1, VEGFA, FGFR1
hsa-miR-183-5p	9.42E-03	37	BRAF, FOS, ITGB1, CDK4, CBL, NRAS, CRKL, STK4, TCF7L2, GNA13, HSP90AA1, ROCK2, TRAF4, IGF1R, GNB1, KRAS, TPM3, TP53, CDH1, AR, CCND1, SMAD4, MSH6, CCNE2, HIF1A, MYC, MSH2, RAC1, FGF2, RALGDS, PTEN, FOXO1, MAPK1, GNG5, PLCB4, CCDC6, RASSF5
hsa-miR-195-5p	9.00E-04	62	DVL3, STAT3, GNA12, CDK4, CXCL8, SMAD2, CRKL, CRK, COL4A5, GNA13, HSP90AA1, GLI2, BDKRB2, FZD6, CHUK, BCL2, TRAF4, BIRC5, IGF1R, GNAI3, PTCH2, TRAF5, CDK6, PML, VHL, IKBKB, ARHGEF11, CDH1, MAPK9, GNB2, CCND1, CTNNB1, CCNE2, E2F3, MAPK8, MYC, MMP9, CBLC, PRKACA, HSP90AB1, FGF2, CDC42, EP300, GNAQ, NKX3-1, AKT3, PIAS2, WNT3A, CCNE1, PIK3CA, CDKN1A, LAMC2, HGF, VEGFA, CREBBP, GRB2, TGF, MDM2, WNT9A, XIAP, PDGFA, ADCY6
hsa-miR-378a-3p	1.57E-02	37	MET, CBL, CRKL, GNAS, TGFA, ARHGEF12, GNA13, HSP90AA1, ROCK2, ARNT, GNG12, BCL2, CXCL12, IGF1R, CDK6, PML, JUN, PIK3R3, CCND1, DVL1, CTNNA1, E2F3, MYC, PIK3R1, HDAC2, EP300, RBX1, SUFU, VEGFA, PTEN, MAPK1, GNG5, GRB2, DVL2, TRAF, MDM2, GNAI1
hsa-miR-497-5p	2.50E-03	56	BRAF, DVL3, SOS2, CDK4, SMAD2, PTCH1, WNT5A, PIK2R2, HSP90AA1, GLI2, ROCK2, FZD6, SMAD3, CHUK, RARB, BIRC5, IGF1R, PTCH2, CDK6, TCEB1, PML, IKBKB, AKT2, MMP2, GNB2, CCND1, LPAR1, MMP1, E2F3, MAPK8, MYC, MMP9, HSP90AB1, NOS2, FGF2, CDC42, LAMC1, AKT3, CCNE1, PIK3CA, FN1, CDKN1A, MAP2K1, SMO, LAMC2, HGF, MTOR, VEGFA, MAPK1, GRB2, MDM2, WNT9A, ADCY6
Central carbon metabolism in cancer (hsa05230)			
hsa-miR-1246	2.06E-02	3	PIK3CB, TP53, PDK1
hsa-miR-133b	8.46E-04	4	MET, PKM, EGFR, FGFR1
hsa-miR-150-5p	2.40E-07	6	MET, PKM, TP53, FLT3, SLC2A1, FGFR1
hsa-miR-375	7.98E-03	9	PDGFRA, ERBB2, FGFR3, RAF1, GLS, HK2, HIF1A, MYC, PDK1
hsa-miR-378a-3p	2.66E-02	10	MET, PKM, GLS, SLC7A5, PIK3R3, MYC, PIK3R1, LDHA, PTEN, MAPK1
FoxO signaling pathway (hsa04068)			
hsa-miR-145-5p	3.31E-02	17	IRS2, STAT3, SMAD2, BNIP3, NRAS, MAPK14, IGF1R, EGFR, TGFB1, ATM, CCND1, AKT1, IRS1, TGFB2, CDKN1A, MDM2, TGFB3

hsa-miR-183-5p	3.16E-02	17	BRAF, FBXO32, CCNB1, NRAS, STK4, PCK2, IGF1R, KRAS, CCND1, SMAD4, IRS4, SOD2, PTEN, SGK3, FOXO1, MAPK1, BCL2L11
hsa-miR-224-5p	2.49E-02	18	NRAS, SETD7, CDKN1B, IGF1R, EGFR, PIK3R3, CCND1, SMAD4, MAPK8, INSR, PRKAA1, MAPK12, SOD2, CDKN1A, PLK3, CCNG2, BCL2L11, TGFB3
hsa-miR-375	7.98E-03	17	PRKAA2, SETD7, CCND2, RAF1, CDKN2B, IGF1R, GADD45A, HOMER2, PLK2, IRS1, TGFB2, SOD2, FOXO3, USP7, CDKN1A, MDM2, BCL2L11
hsa-miR-497-5p	2.50E-03	28	BRAF, CCNB1, SOS2, SMAD2, PRKAA2, PIK3R2, CCND2, STK11, SMAD3, CHUK, IGF1R, IKBKB, AKT2, NLK, CCND1, S1PR1, MAPK8, IRS4, PRKAB2, AKT3, PIK3CA, CDKN1A, MAP2K1, MAPK1, GRB2, SGK1, MDM2, BCL2L11
TGF-beta signaling pathway (hsa04350)			
hsa-miR-145-5p	1.04E-03	12	FST, SMAD2, NODAL, THBS1, ID4, TGFB1, ACVR1, MYC, BPM7, TGFB3
hsa-miR-150-5p	2.36E-04	5	SMAD3, BPM2, SP1, EP300, PPP2CB
hsa-miR-18b-5p	2.81E-02	4	SMAD2, ACVR2B, ACVR2A, PPP2R1B
hsa-miR-195-5p	2.22E-03	16	SMAD2, PPP2CA, SMURF2, CUL1, MYC, PPP2R1A, RBL1, ACVR2A, SP1, EP300, PPP2CB, BAMBI, LTBP1, SMAD7, CREBBP, PPP2R1B
hsa-miR-497-5p	8.20E-04	13	FST, SMAD2, PPP2CA, ACVR1B, SMURF2, SMAD3. ID4, ACVR2B, MYC, RBL1, SP1, SMAD7, MAPK1
Colorectal cancer (hsa05210)			
hsa-miR-145-5p	2.22E-03	10	GSK3B, SMAD2, TGFB1, CCND1, CTNNB1, AKT1, MYC, RAC1, TGFB2, TGFB3
hsa-miR-183-5p	2.99E-03	13	BRAF, FOS, TCF7L2, KRAS, TP53, CCND1, SMAD4, MSH6, MYC, MSH2, RAC1, RALGDS, MAPK1
hsa-miR-21-5p	2.14E-05	17	TGFBR1, APC, BCL2, BIRC5, AXIN1, APPL1, TGFB1, AKT2, CCND1, CTNNB1, MSH6, MYC, MSH2, PIK3R1, TGFB2, MAPK1, TGFBR2
hsa-miR-497-5p	1.94E-02	14	BRAF, SMAD2, PIK3R2, SMAD3, BCL2, BIRC5, AKT2, CCND1, MAPK8, MYC, AKT3, PIK3CA, MAP2K1, MAPK1
Cell cycle (hsa04110)			
hsa-miR-143-3p	3.82E-02	13	SMC1A, CDK4, DBF4, YWHAB, WEE1, CDK6, YWHAQ, TFDP2, MCM2, CDC7, EP300, MDM2, MCM3
hsa-miR-195-5p	4.65E-03	29	RBL2, CDK4, SMAD2, CDC14A, CCNA2, YWHAG, CCND2, SMC3, CUL1, YWHAB, WEE1, CDK6, CCND1, CCNE2, E2F3, MYC, RBL1, TTK, CDC7, EP300, CCNE1, CDC27, CDKN1A, PRKDC, ANAPC13, CREBBP, MDM2, CCND3, CDC25A
hsa-miR-21-5p	1.18E-03	25	E2F1, SMC1A, E2F2, MDM6, CCND2, BUB1, MCM4, STAG2, WEE1, CDK6, TGFB1, TFDP2, CCND1, SKP2, E2F3, MYC, RB1, HDAC2, ANAPC5, TGFB2, PRKDC, PLK1, MDM2, MCM3, CDC25A
hsa-miR-378a-3p	1.19E-02	20	CCNB1, YWHAG, CCND2, MCM4, CDK1, CDK6, BUB3, CHEK1, SMC1B, CCND1, E2F5, E2F3, MYC, YWHAZ, HDAC2, EP300, RBX1, PLK1, RAD21, MDM2

ErbB signaling pathway (hsa04012)			
hsa-miR-145-5p	6.09E-03	12	GSK3B, PRKCA, NRAS, EGFR, ERBB3, PAK1, PTK2, AKT1, MYC, PAK4, CDKN1A, ABL2
hsa-miR-21-5p	2.75E-02	14	ERBB2, SOS2, NRAS, CRKL, PAK2, MAP2K7, EGFR, AKT2, PTK2, MYC, PIK3R1, GAB1, MAP2K4, MAPK1
hsa-miR-378a-3p	2.01E-02	14	CBL, CRKL, MAP2K7, TGFA, JUN, PIK3R3, MYC, PIK3R1, PAK4, NRG1, MAPK1, ABL2, GRB2, ELK1
HIF-1 signaling pathway (hsa04066)			
hsa-miR-150-5p	2.96E-02	6	CDKN1B, TCEB2, EP300, EIF4E2, SLC2A1, VEGFA
hsa-miR-21-5p	8.99E-03	22	STAT3, ERBB2, NFKB1, CUL2, GAPDH, BCL2, IGF1R, EGFR, TRL4, HK2, AKT2, PDHA2, TFRC, PIK3R1, LDHA, PFKFB2, MKNK2, ALDOA, VEGFA, MAPK1, TEK, PGK1
hsa-miR-378a-3p	2.01E-02	16	IFNGR2, ARNT, BCL2, IGF1R, TLR4, ENO1, PIK3R3, TFRC, PIK3R1, LDHA, EP300, RBX1, ALDOA, VEGFA, MAPK1, SERPINE1
mTOR signaling pathway (hsa04150)			
hsa-miR-223-3p	2.58E-02	4	DDIT4, PIK3CD, MTOR, VEGFA
hsa-miR-497-5p	3.44E-04	18	BRAF, RRAGD, PRKAA2, PIK3R2, STK11, RPS6, RICTOR, IKBKB, AKT2, EIF4E, RRAGA, RPS6KA3, AKT3, PIK3CA, MTOR, ULK1, VEGFA, MAPK1
MicroRNAs in cancer (hsa05206)			
hsa-miR-143-3p	3.80E-02	23	THBS1, MAPK7, BCL2, GLS, KRAS, CDK6, KIF23, PRKCE, SLC7A1, RPTOR, PIM1, PTGS2, IRS1, DNMT3A, FSCN1, HRAS, EP300, CYP1B1, PTEN, MAPK1, PLAU, MDM2, CD44
hsa-miR-150-5p	1.21E-04	8	MET, CDKN1B, ZEB1, TP53, MDM4, EP300, NOTCH3, VEGFA
PI3K-Akt signaling pathway (hsa04151)			
hsa-miR-145-5p	6.09E-03	38	GSK3B, PRKCA, ITGB1, ITGB8, CDK4, GNG11, NRAS, THBS1, IFNB1, ANGPT2, ITGA5, ITGA3, EGFR, COL3A1, COL6A1, PTK2, ITGAV, ITGA11, DDIT4, CCND1, EIF4E, AKT1, MYC, COL5A1, COL1A1, HSP90AB1, IRS1, RAC1, COL1A2, FN1, TNC, CDKN1A, VEGFA, COL5A2, SPP1, MDM2, PDGFRB
hsa-miR-497-5p	3.08E-02	48	SOS2, CDK4, ATF2, PRKAA2, PPP2CA, PIK3R2, IL2RB, MCL1, CCND2, HSP90AA1, STK11, CHUK, BCL2, IGF1R, RPS6, CDK6, IKBKB, COL6A1, AKT2, CRTC2, GNB2, CCND1, EIF4E, LPAR1, MYC, HSP90AB1, HSP90B1, FGF2, COL1A2, LAMC1, AKT3, CCNE1, PIK3CA, FN1, TNC, CDKN1A, MAP2K1, LAMC2, HGF, MTOR, COL5A3, VEGFA, MAPK1, GRB2, SGK1, MDM2, BCL2L11, CCND3

Overall, the two approaches here adopted demonstrated the strong involvement of the selected miRNAs in the pathogenetic mechanisms underlying the

development of CRC. Through these analyses it was also demonstrated that the CRC-associated miRNAs were able to target a plethora of genes with key functions in cellular homeostasis, cell proliferation and neoplastic transformation.

These findings suggest how these miRNAs may serve not only as novel diagnostic biomarkers, but also as novel tumor-regulators that can be potentially targeted to inhibit cancer development, cell survival, proliferation and epithelial-to-mesenchymal transition.

By deeply analyzing these computational results, four miRNAs emerged as strongly dysregulated and strongly involved in CRC. Therefore, further validation analyses on CRC clinical samples were performed on a subset of four miRNAs, of which two were up-regulated: hsa-miR-21-5p and hsa-miR-503-5p; while the other two were down-regulated: hsa-miR-375 and hsa-miR-497-5p. The validation analyses were performed by using ddPCR absolute quantification.

4.2 Clinical validation of the diagnostic role of the four selected miRNAs in Colorectal Cancer samples

To validate the diagnostic potential of the four selected miRNAs hsa-miR-21-5p, hsa-miR-503-5p, hsa-miR-375 and hsa-miR-497-5p the expression levels of miRNAs were firstly evaluated on FFPE tissue samples obtained from CRC patients. For each patient, both tumor and normal adjacent FFPE tissues were obtained in order to evaluate differential expression between these two groups.

All the analyses were performed by using the custom Qiagen-Bio-Rad ddPCR protocol developed at the Laboratory of Experimental Oncology of the University of Catania.

For all the four selected miRNAs and for the U6 snRNA used as internal control very good ddPCR amplification signals were obtained as shown in Figure 12.

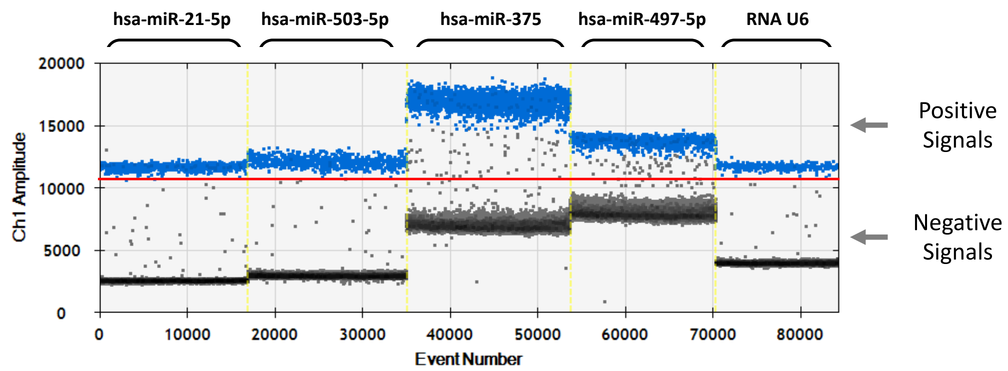

Figure 12. ddPCR amplification signals of the four selected miRNAs and the endogenous control U6 snRNA.

The ddPCR analyses were thus performed in the case series of CRC patients recruited for this study by comparing the expression levels of miRNAs obtained in FFPE CRC tissue samples and in normal adjacent mucosa. The ddPCR analyses revealed statistically significant results obtained for all the analyzed miRNAs which were significantly modulated in CRC tissue samples vs normal control thus confirming the high predictive value of the computational analyses previously conducted.

In particular, the expression levels of the two predicted up-regulated miRNAs hsa-miR-21-5p and hsa-miR-503-5p were significantly up-regulated in CRC tissues compared to the normal counterparts ($p<0.0001$) (Figure 13). Similarly, also the expression levels of the down-regulated miRNAs hsa-miR-375 and hsa-miR-497-5p were significantly reduced in CRC samples compared to normal mucosa ($p=0.0073$ and $p=0.0064$, respectively) (Figure 13).

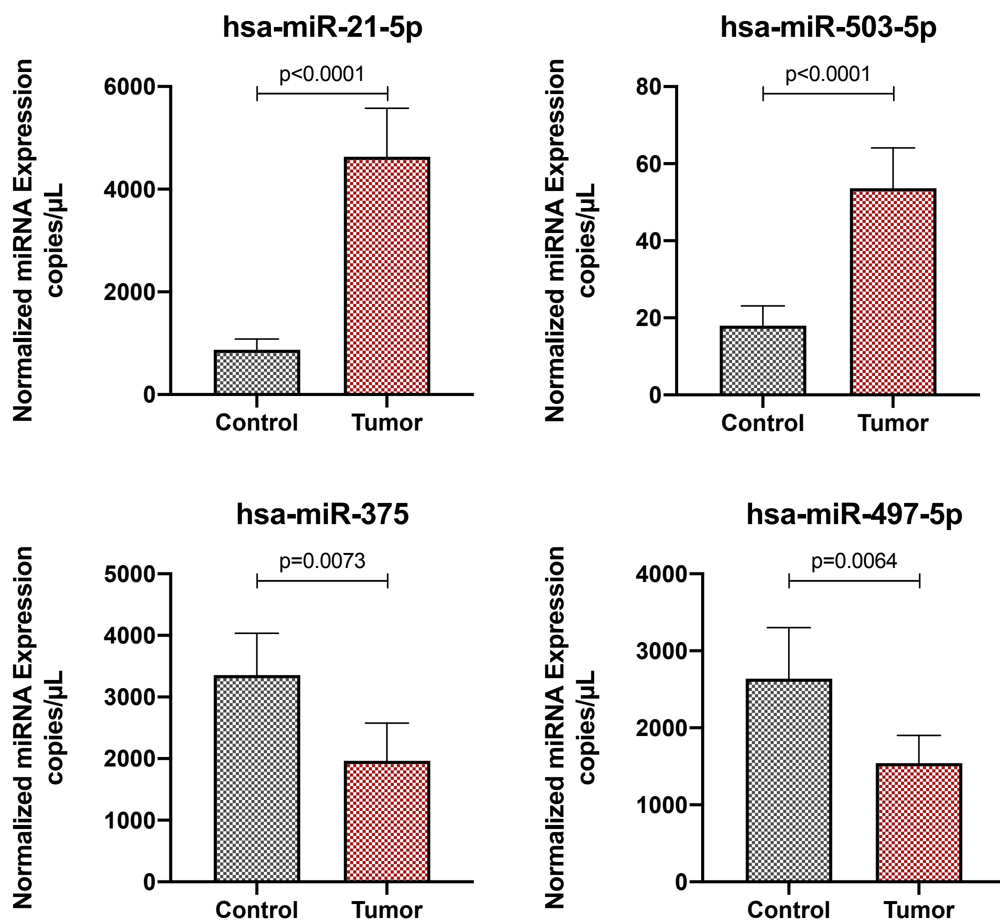

Figure 13. Expression levels of the four selected miRNAs in CRC FFPE tissue and adjacent normal mucosa. The data were considered statistically significant for $p<0.05$.

Overall, the data obtained on FFPE tissue samples confirmed the bioinformatics analyses suggesting that the evaluation of miRNA expression levels in CRC biopsies could be indicative of the presence of tumor.

From a statistical point of view, the diagnostic potential of the four selected miRNAs was evaluated by performing ROC analyses. These analyses further confirmed the diagnostic value of the selected miRNAs demonstrating a very good diagnostic accuracy for the two up-regulated miRNAs hsa-miR-21-5p and hsa-miR-503-5p with AUC values of 0.8925 ($p<0.0001$) and 0.8350 ($p=0.0003$), respectively (Figure 14). As regards the two down-regulated miRNAs, only hsa-miR-375 showed a low-moderate diagnostic accuracy with an AUC value of 0.7200 ($p<0.0173$) while no significant data were obtained for hsa-miR-497-5p (AUC=0.6550, $p=0.0935$) (Figure 14).

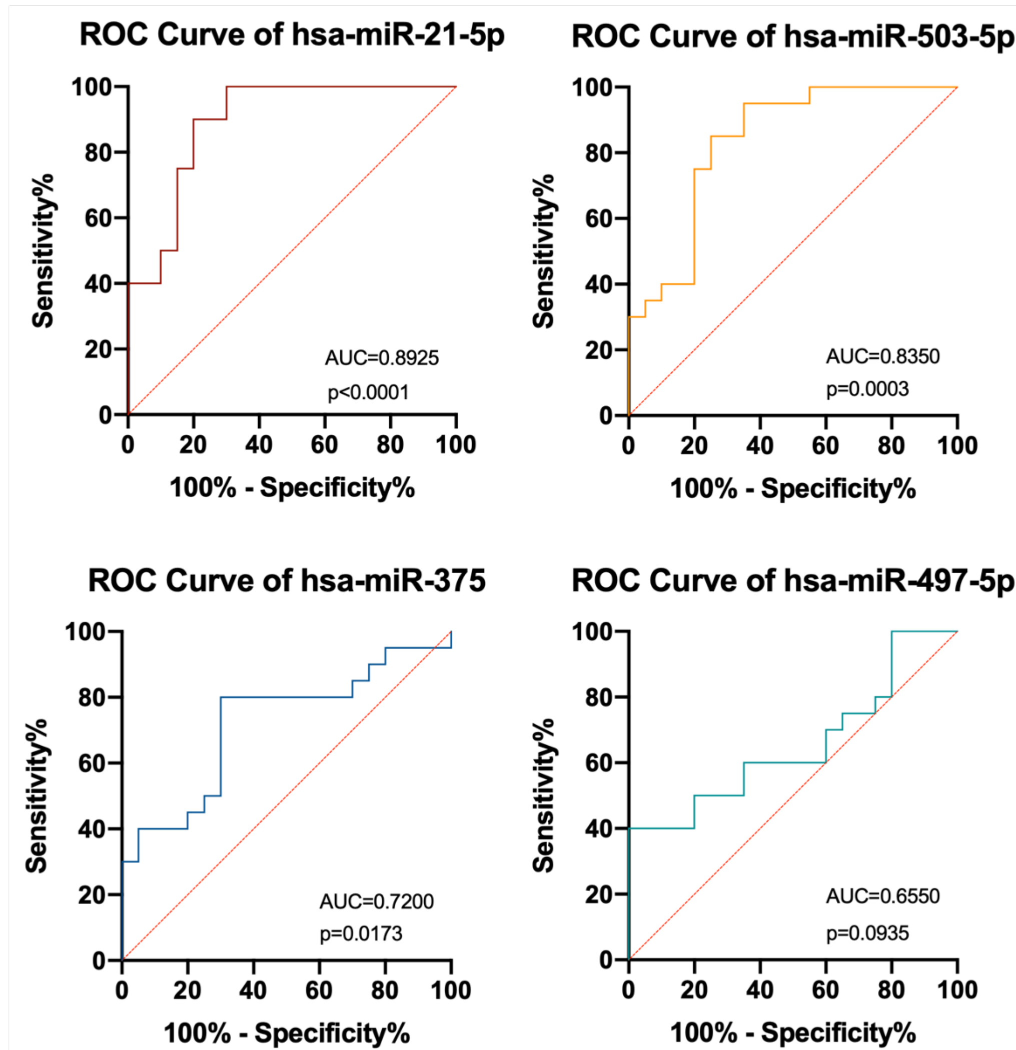

Figure 14. Diagnostic potential of the four selected miRNAs analyzed in FFPE samples according to ROC curves analysis. The data were considered statistically significant for $p<0.05$.

Overall, for the four selected miRNAs, the following sensitivity and specificity values were obtained:

- hsa-miR-21-5p: Sensitivity=90%; Specificity=80%;
- hsa-miR-503-5p: Sensitivity=95%; Specificity=75%;
- hsa-miR-375: Sensitivity=80%; Specificity=70%;
- hsa-miR-497-5p: not statistically significant data.

In order to propose these four miRNAs as non-invasive biomarkers for the early diagnosis of CRC and for the development of novel screening strategies for individuals at risk for this tumor, the expression levels of the four selected miRNAs were evaluated in liquid biopsy samples obtained from CRC patients

and healthy donors by using the ddPCR protocol previously described. Therefore, the expression levels of the four miRNAs were evaluated in 14 patients with CRC and 10 healthy donors.

The results obtained through these analyses were less evident compared to those obtained in FFPE samples. Indeed, the absolute quantification performed by ddPCR in liquid biopsy samples demonstrated a significant dysregulation of miRNAs only for the miRNAs hsa-miR-21-5p and hsa-miR-497-5p that were, respectively up- and down-regulated in CRC samples compared to healthy donors ($p=0.0408$ and $p=0.0164$, respectively). On the contrary, no significant data were obtained for the miRNAs hsa-miR-503-5p and hsa-miR-375 (Figure 15).

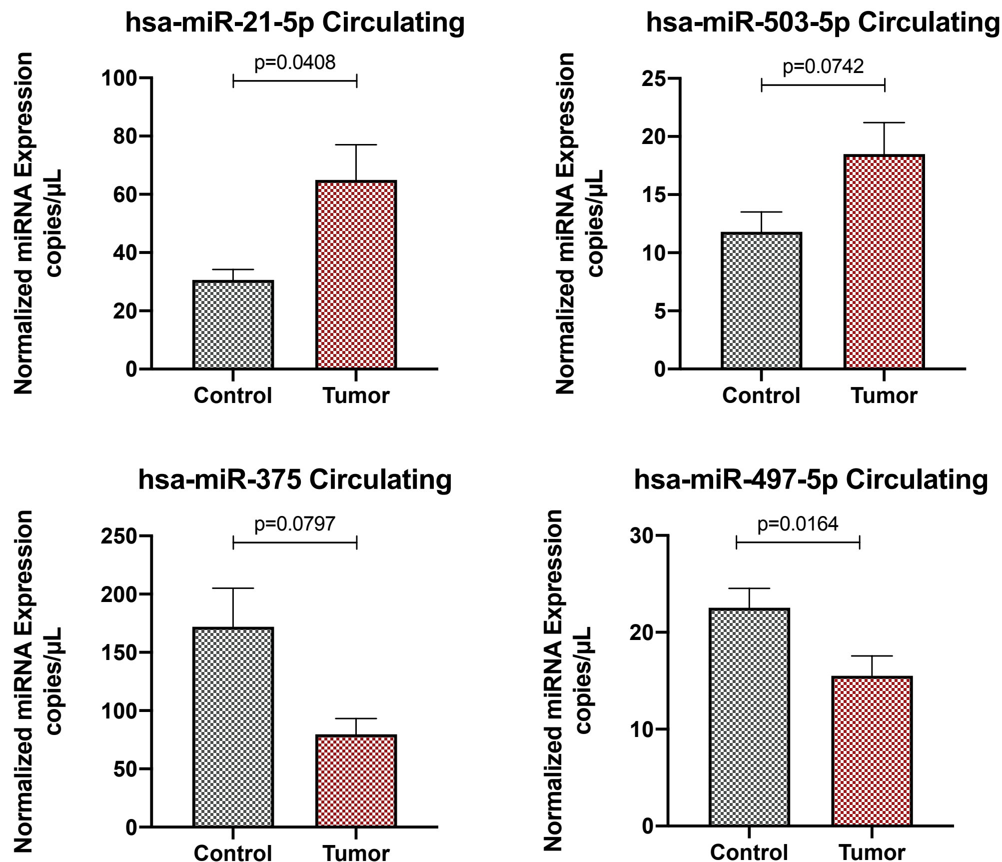

Figure 15. Expression levels of the four selected miRNAs in liquid biopsy samples obtained from CRC patients and healthy donors. The data were considered for $p<0.05$.

Of note, the less relevant results obtained for liquid biopsy samples may be due to the low number of samples analyzed in the study. Therefore, the data should be confirmed in a wider cohort of liquid biopsy samples. In addition, another

possible reason behind the less significant results obtained in serum samples may be due to the dilution of CRC-related miRNAs with miRNAs deriving from other tissue and organs, therefore, further investigations are needed to isolate and evaluate only tumor-related circulating miRNAs.

Accordingly, the ROC analyses revealed a weaker diagnostic potential of the selected miRNAs when analyzed in liquid biopsy samples. Only the ROC curves obtained for hsa-miR-21-5p (AUC=0.7200) and hsa-miR-497-5p (AUC=0.7556) were statistically significant (p=0.0401 and p=0.0171, respectively), while no significant data were obtained from the analyses of the other two miRNAs, however, very low AUC values were obtained (Figure 16).

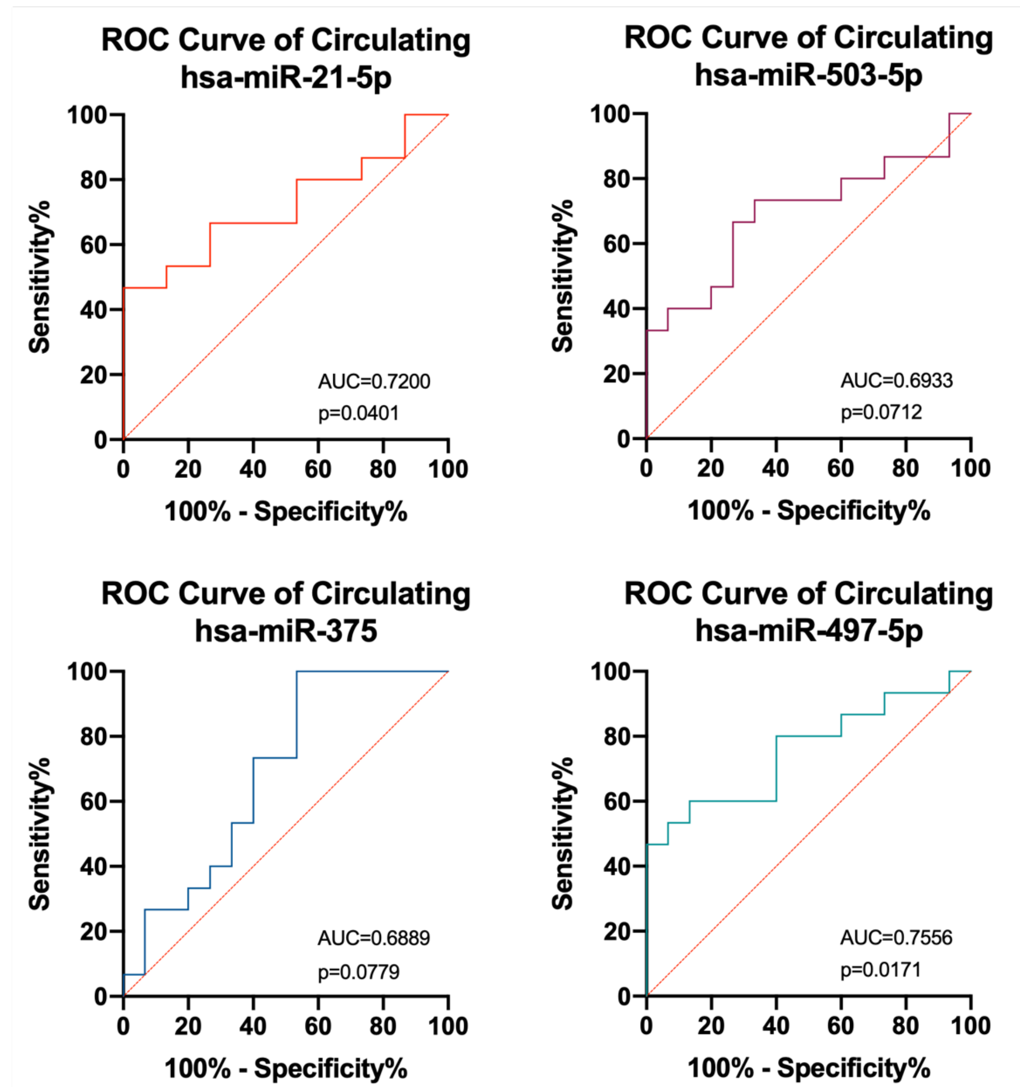

Figure 16. Diagnostic potential of the four selected miRNAs analyzed in liquid biopsy samples according to ROC curves analysis. The data were considered statistically significant for $p<0.05$.

Overall, the sensitivity and specificity rates observed in FFPE samples were higher than those observed in liquid biopsy samples.
The ddPCR results obtained from both FFPE and liquid biopsy samples confirmed the good predictive value of the computational analyses performed and the potential clinical utility of the evaluation of miRNAs expression level for the early diagnosis of CRC.

4.3 Bioinformatics evaluation of the prognostic value of the four selected miRNAs

Due to the lack of follow-up data on the patients recruited in the study, further computational analyses were performed on the miRNA expression data and clinical-pathological data contained in the TCGA COADREAD database to evaluate the prognostic value of the selected and validated miRNAs.
First, the genes directly modulated by all the four selected miRNAs were identified by using miRTargetLink Human software. The miRTargetLink Human analysis revealed that the selected miRNAs can alter the expression levels of a total of ten different genes with strong interaction levels (Figure 17).

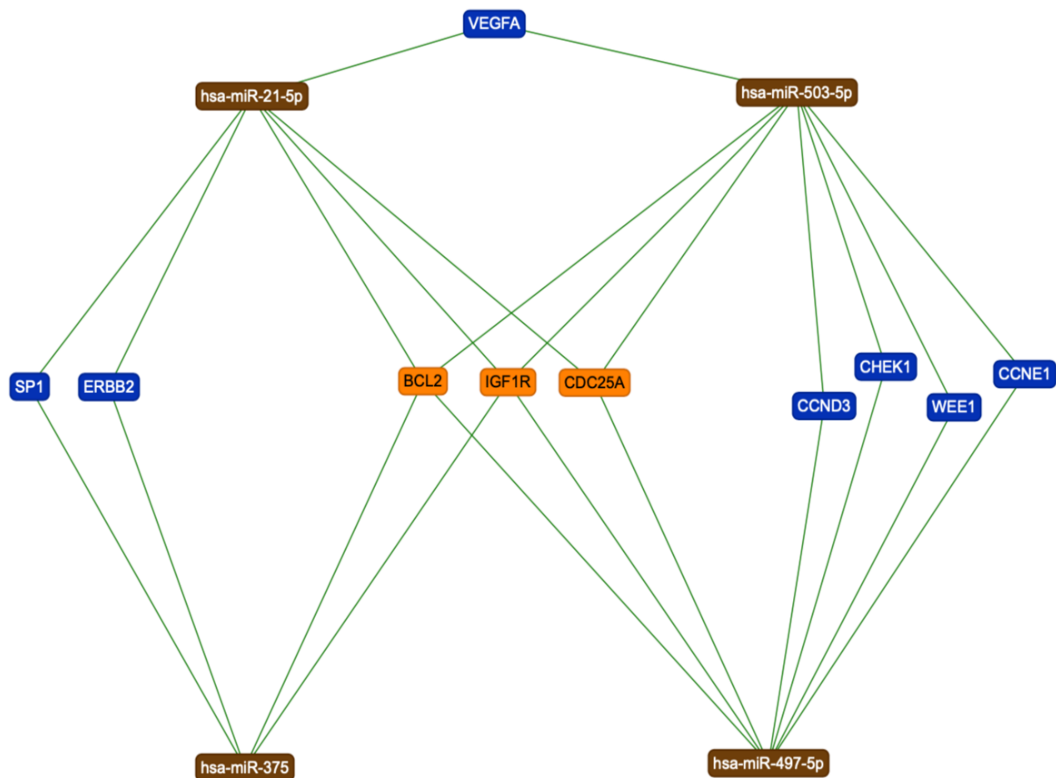

Figure 17. Interaction network between the four selected miRNAs and their targeted genes according to miRTargetLink Human analysis.

Only three out of ten genes were targeted by all the four miRNAs: BCL2, IGF1R and CDC25A suggesting that the four miRNAs epigenetically modulate the expression levels of these three genes notoriously involved in neoplastic transformation. Other interesting data are related to VEGFA that was targeted by the two up-regulated miRNAs hsa-miR-21-5p and hsa-miR-503-5p, while no genes were in common between the two down-regulated miRNAs. As regards the remaining six genes, two were shared by hsa-miR-21-5p and hsa-miR-375: SP1 and ERBB2; while the other four were in common between hsa-miR-503-5p and hsa-miR-497-5p: CCND3, CHEK1, WEE1 and CCNE1.

To further establish the protein-protein interactions between these ten miRNA-modulated genes, the STRING analysis was performed. The strong interconnection between these genes is shown in Figure 18.

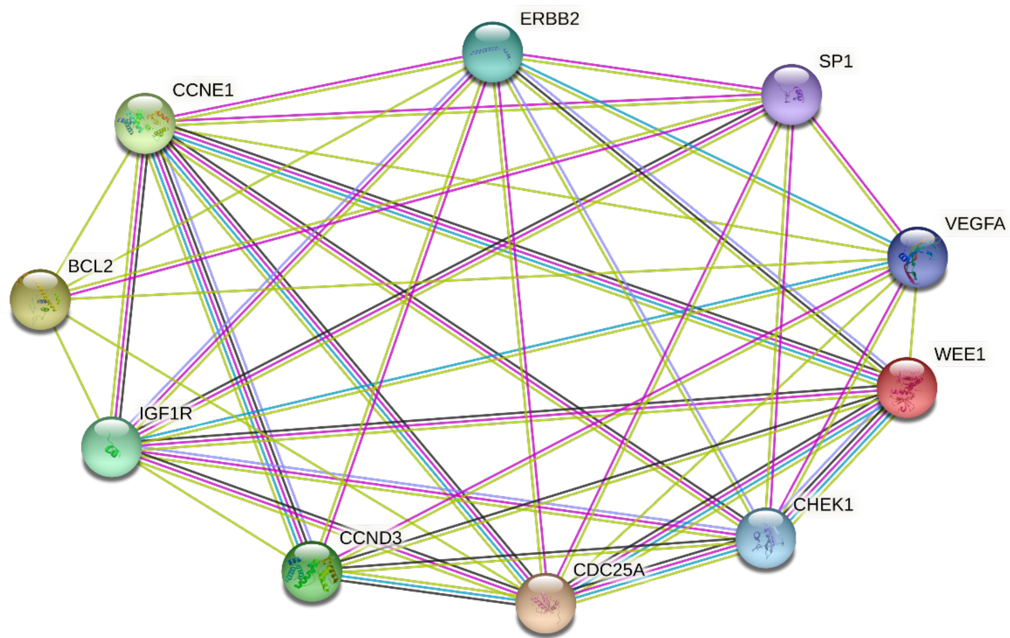

Figure 18. STRING protein-protein interaction network between the ten genes strongly modulated by the four validated miRNAs

Among the ten genes, WEE1, CHEK1, CDC25A, CCND3, IGF1R and CCNE1 were those which form the more complex network.

Subsequently, a GO Panther analysis was performed to assess the functional role of these genes and in particular their involvement in biological processes, the protein class and the molecular pathways in which these genes and their modulator miRNAs are involved.

As regards the molecular functions performed by the ten miRNA-targeted genes, five major molecular functions were identified and in particular, binding activity, catalytic activity, molecular function regulator, molecular transducer activity and transcription regulator activity (Figure 19).

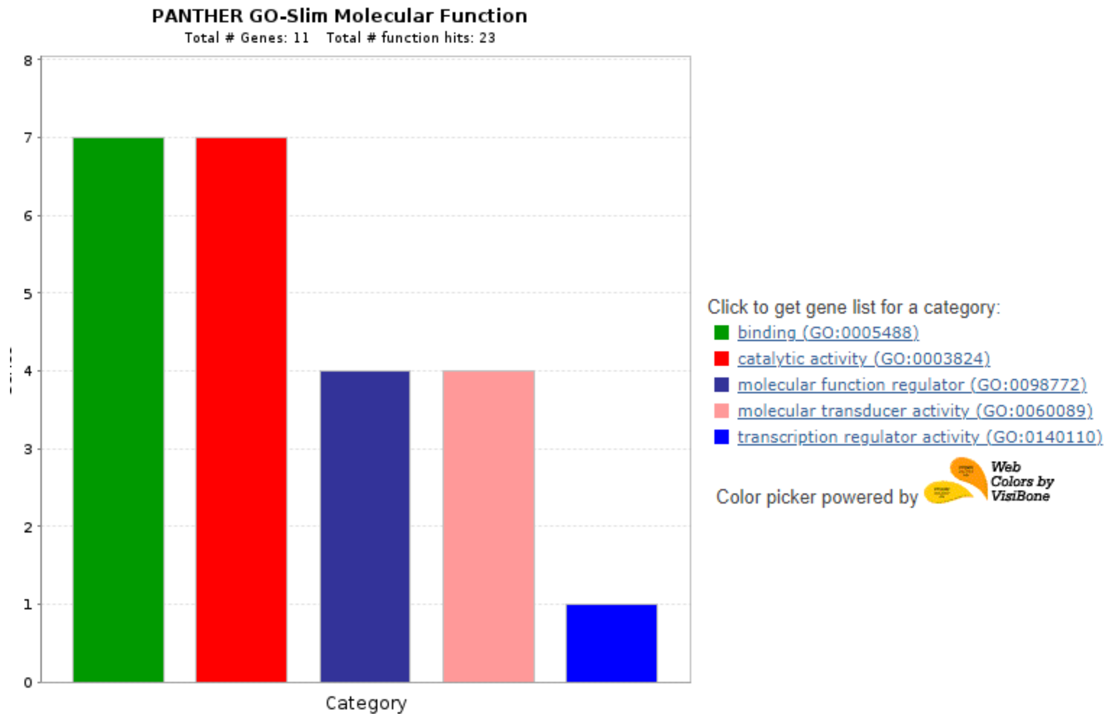

Figure 19. Molecular functions of the ten genes strongly modulated by the four validated miRNAs according to GO PANTHER analysis.

These data suggest how the four miRNAs are involved in the modulation of genes that in turn are responsible for the regulation of several other mediators involved in CRC development.

By analyzing the protein class of the miRNA-targeted genes, other five groups were identified: more in detail, 3 genes are protein modifying enzymes; 2 are protein-binding activity modulators; 2 are transmembrane signal receptor; 1 is a gene-specific transcriptional regulator and 1 is an intercellular signal molecule (Figure 20).

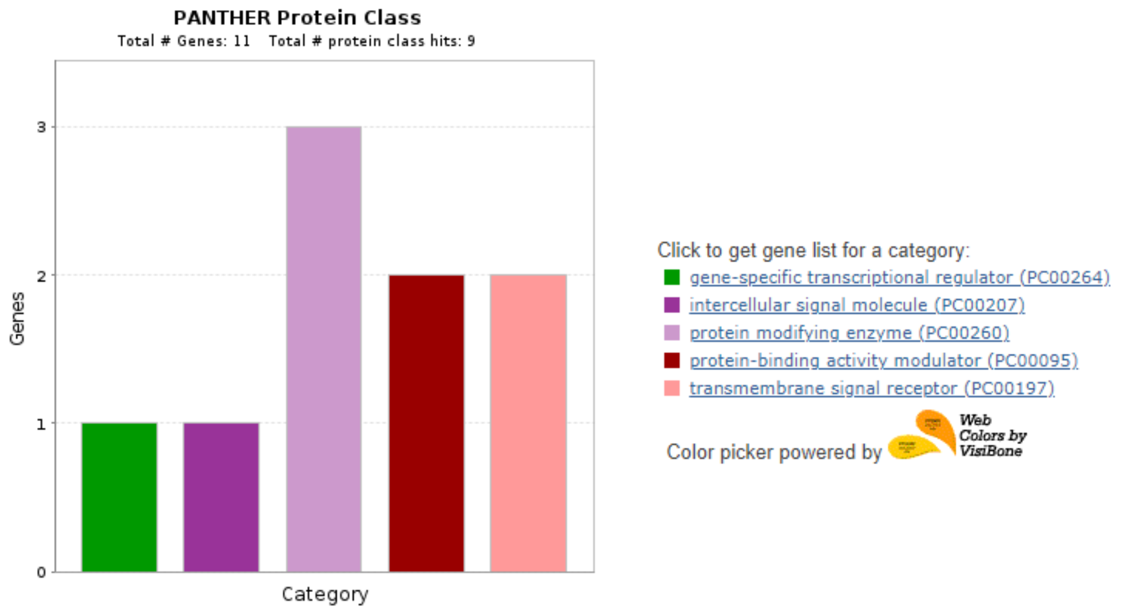

Figure 20. Protein class of the ten genes strongly modulated by the four validated miRNAs according to GO PANTHER analysis.

Subsequently the biological processes were investigated. As displayed in Figure 21, the ten genes are involved in a wide variety of biological processes, among which the biological regulation, cellular and metabolic processes were more represented. Also the response to stimuli and signaling processes were widely modulated by miRNAs and genes (Figure 21).

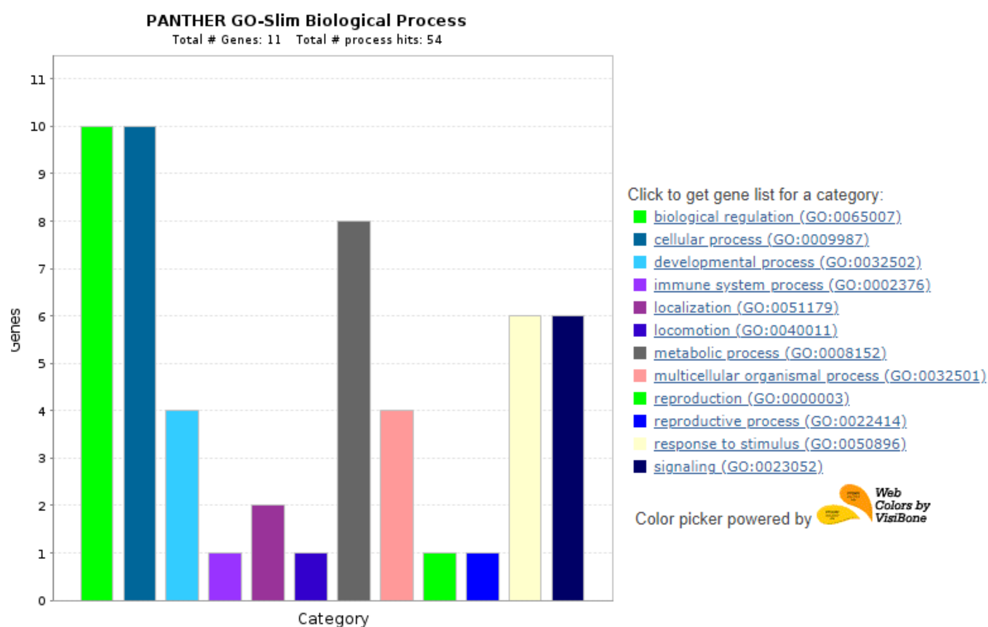

Figure 21. Biological processes of the ten genes strongly modulated by the four validated miRNAs according to GO PANTHER analysis.

The latter Figure shows the association between the ten highlighted genes and the pathways in which they are involved. The majority of the genes are involved in cell cycle-related pathways, p53 pathways, gonadotropin-releasing pathway and CCKR signaling map, thus confirming the results obtained in the preliminary DIANA-mirPath analyses (Figure 22).

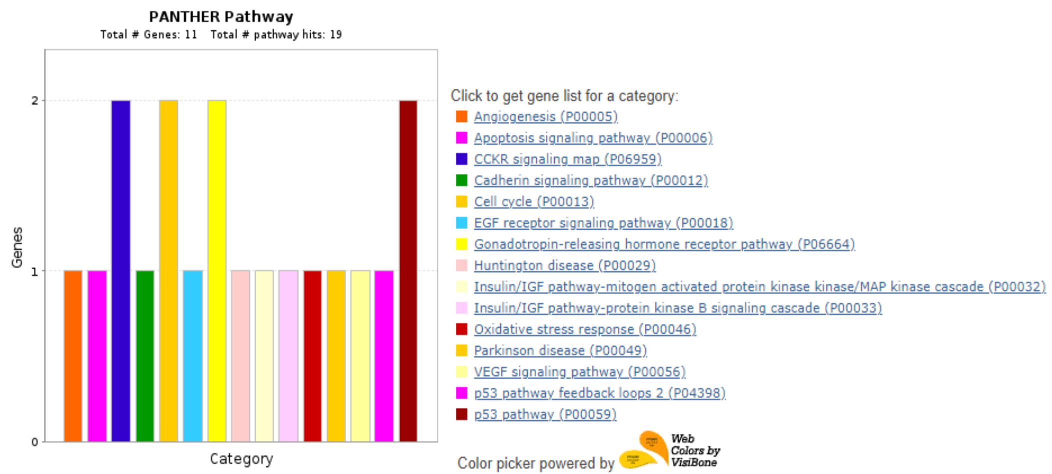

Figure 22. Molecular pathways of the ten genes strongly modulated by the four validated miRNAs according to GO PANTHER analysis.

All the GO Panther analyses revealed how the four miRNAs are capable to alter a wide variety of pathways and cellular-molecular processes by the modulation of the expression levels of the ten genes which they strongly regulate.

Finally, GEPIA and OncoLnc analyses were performed to evaluate the effective role and alteration of the ten miRNA-targeted genes in CRC and to evaluate the prognostic significance of the selected miRNAs. Of note, both bioinformatics tools use the data deposited in the TCGA COADREAD database.

As regards the analyses performed with GEPIA, the results showed that five of the ten genes have dysregulated expression levels in CRC (Figure 23).

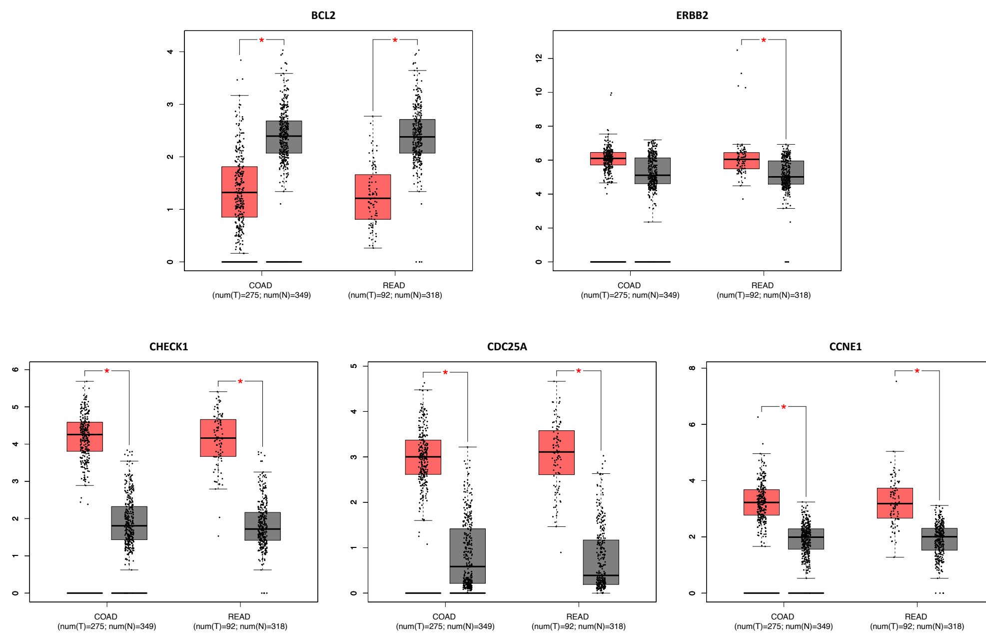

Figure 23. GEPIA analysis of the expression levels of miRNA-targeted genes in COAD and READ patients compared with healthy controls, according to TCGA and GTEx databases.

The GEPIA analysis highlighted that BCL2 expression levels were down-regulated in COAD (Colon cancer patients) and in READ (Rectal cancer patients) databases compared to healthy controls contained in the GTEx database. CHEK1, CDC25A and CCNE1 showed a significantly higher expression both in COAD and READ patients compared to healthy controls. Contrarily, ERBB2 expression levels followed a different pattern: a slight increase was observed for READ patients, but not for COAD ones, compared to healthy controls (Figure 23).

Coming back to the prognostic role of the four selected and validated miRNAs hsa-miR-21-5p, hsa-miR-503-5p, hsa-miR-375 and hsa-miR-497-5p, the OncoLnc tool was used to unveil the prognostic significance of miRNAs in predicting the overall survival of CRC patients. The results of such analyses performed on the data contained in the TCGA COAD database revealed that dysregulation of hsa-miR-21-5p, hsa-miR-503-5p and hsa-miR-375 as predicted by computational investigations and validated through ddPCR are associated with a worse overall survival (Figure 24).

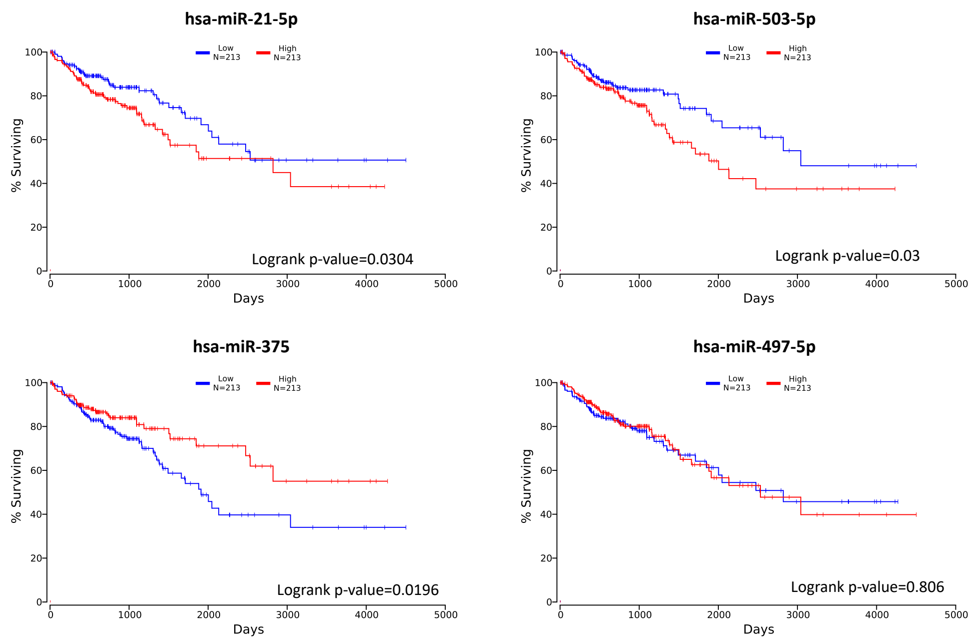

Figure 24. Prognostic value of the four selected and validated miRNAs according to the OncoLnc analysis performed on TCGA COAD database.

The same OncoLnc analysis performed for patients with rectal cancers contained in the TCGA READ database revealed that only the down-regulated hsa-miR-375 can be considered a prognostic biomarker to define the overall survival of rectal cancer patients (Figure 25).

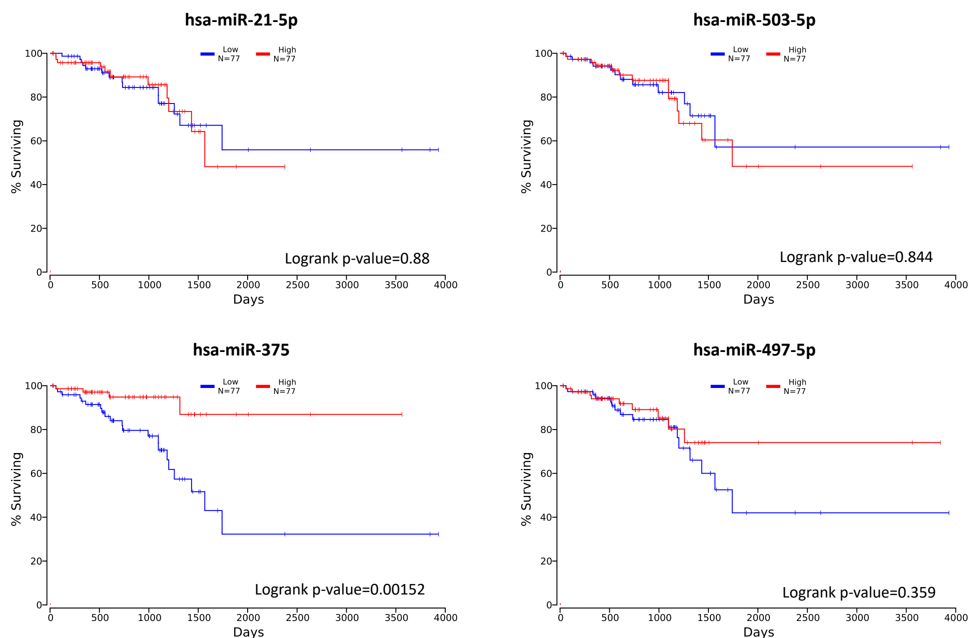

Figure 25. Prognostic value of the four selected and validated miRNAs according to the OncoLnc analysis performed on TCGA READ database.

No significant data were obtained for the other three miRNAs. Noteworthy, although not statistically significant, hsa-miR-497-5p shows a trend of better survival when overexpressed.

4.4 Positive miRNA modulation induced by microbial interventions with the administration of probiotics in CRC patients

The computational and experimental results described in the above chapters highlighted how the four selected miRNAs can be used as diagnostic biomarkers for the early identification of CRC as well as prognostic biomarkers to predict the survival of patients. However, no investigations on the epigenetic modulators of the expression levels of these miRNAs were performed.

For this purpose, in the present study both computational and experimental investigations were performed to evaluate the modulatory effects of microbial interventions in CRC patients treated with probiotics during anticancer therapies.

Therefore, similarly to what was done for the identification of miRNAs associated with the development of colorectal cancer, differential analyses of the expression levels of miRNAs were performed in samples of colonic mucosa treated with probiotics and faecal transplant compared to untreated colonic mucosa (dataset GSE140338).

Differential analyses performed through GEO2R have shown how probiotics interventions aimed at modifying the composition of the intestinal microbiota (enrichment with probiotics or by fecal transplantation) determine a profound alteration of miRNA expression levels. Specifically, 829 miRNAs were found to be significantly up-regulated or down-regulated in the colonic mucosa after administration of probiotics and faecal transplantation ($p<0.05$) (Figure 26).

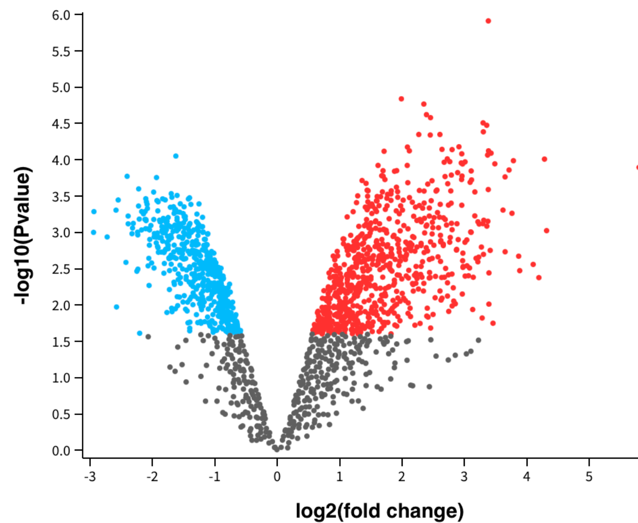

Figure 26. Volcano plot of differential miRNA expression in colonic mucosa treated and untreated with probiotics and faecal transplantation.

In order to identify the miRNAs most modulated by probiotics or faecal transplantation, only the dysregulated miRNAs with p-value lower than $p<0.01$ were taken into consideration. In this way, 518 highly dysregulated miRNAs were identified after microbial interventions with probiotics and fecal transplantation (data not shown).

Subsequently, the list of miRNAs dysregulated in colorectal cancer was merged with the list of miRNAs modulated by the intestinal microbiota to establish which miRNAs involved in CRC development and progression may be positively modulated by microbial interventions.

Through this analysis it was possible to identify 8 common miRNAs, namely hsa-miR-503-5p, hsa-miR-18b-5p, hsa-miR-223-3p, hsa-miR-143-3p, hsa-miR-375, hsa-miR-133b, hsa-miR-21-5p and hsa-miR-145-5p (Figure 27).

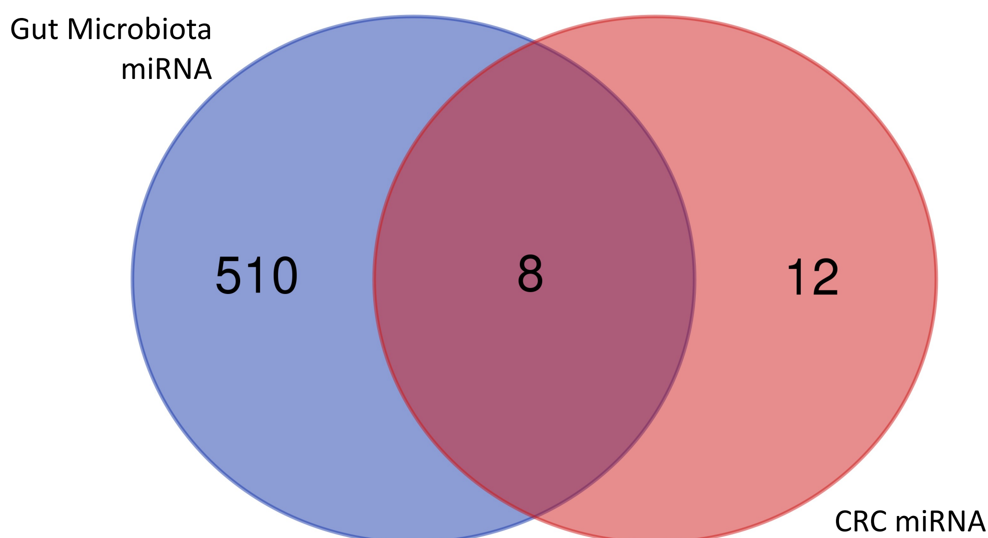

Figure 27. miRNAs modulated by the intestinal microbiota and involved in the development of colorectal cancer.

More interesting data were obtained by analyzing the up-regulation and down-regulation values obtained for these 8 miRNAs shared by the two analyses. More in detail, by comparing the average of the Log2 fold change values obtained for the CRC datasets with those obtained for the gut microbiota dataset, it is possible to observe how for 7 miRNAs, hsa-miR-133b, hsa-miR-145-5p, hsa-miR-503-5p, hsa-miR-18b-5p, hsa-miR-375, hsa-miR-21-5p and hsa-miR-223-3p, the administration of probiotic and faecal transplantation results in a modulation of the expression levels of these miRNAs opposite to the expression found in the tumor. This translates into a potential beneficial effect of probiotics and faecal transplantation which induce a reduction or increase of the expression levels of miRNAs which in the tumor are respectively increased or decreased (Figure 28).

miRNA ID	Log2FC CRC	Log2FC gut microbiota
hsa-miR-133b	**-2.303**	**1.766**
hsa-miR-143-3p	-1.505	-1.400
hsa-miR-145-5p	**-1.667**	**2.014**
hsa-miR-18b-5p	**1.011**	**-1.649**
hsa-miR-21-5p	**2.729**	**-0.915**
hsa-miR-223-3p	**3.358**	**-1.838**
hsa-miR-375	**-1.661**	**1.425**
hsa-miR-503-5p	**1.234**	**-1.198**

Figure 28. Levels of over-expression or down-regulation of miRNAs involved in CRC and modulated by the gut microbiota. In bold the miRNAs positively modulated by the microbiota and showing inverse expression levels compared to the expression normally found in the tumor.

All these bioinformatic data support the hypothesis that the intestinal microbiota is able to actively induce epigenetic modulations of the expression levels of specific miRNAs involved in the development and progression of colorectal cancer.

In order to validate the real epigenetic potential of the intestinal microbiota modifications, the expression levels of three of the eight miRNAs identified here were validated on a pilot case series of liquid biopsy samples obtained from patients affected by CRC and recruited within the two clinical studies based on the administration of LGG in cancer patients undergoing radiotherapy and chemotherapy. The selected miRNAs were hsa-miR-21-5p, hsa-miR-375 and hsa-miR-503-5p.

The evaluation of the epigenetic modulation induced by microbiota enrichment on the expression levels of the three selected miRNAs was performed on liquid biopsy samples collected from 14 CRC patients before and after the administration of LGG as microbiota modulators.

This analysis reveals how the treatment with LGG induces a modulation of miRNA expression levels. In particular, the up-regulated miRNAs in colorectal cancer, i.e. hsa-miR-21-5p and hsa-miR-503-5p, showed a significant down-regulation after probiotics administration (significant data obtained only for hsa-miR-21-5p); conversely, the down-regulated miRNA in colorectal cancer hsa-miR-375, were found to be up-regulated following treatment with LGG (Figure 29).

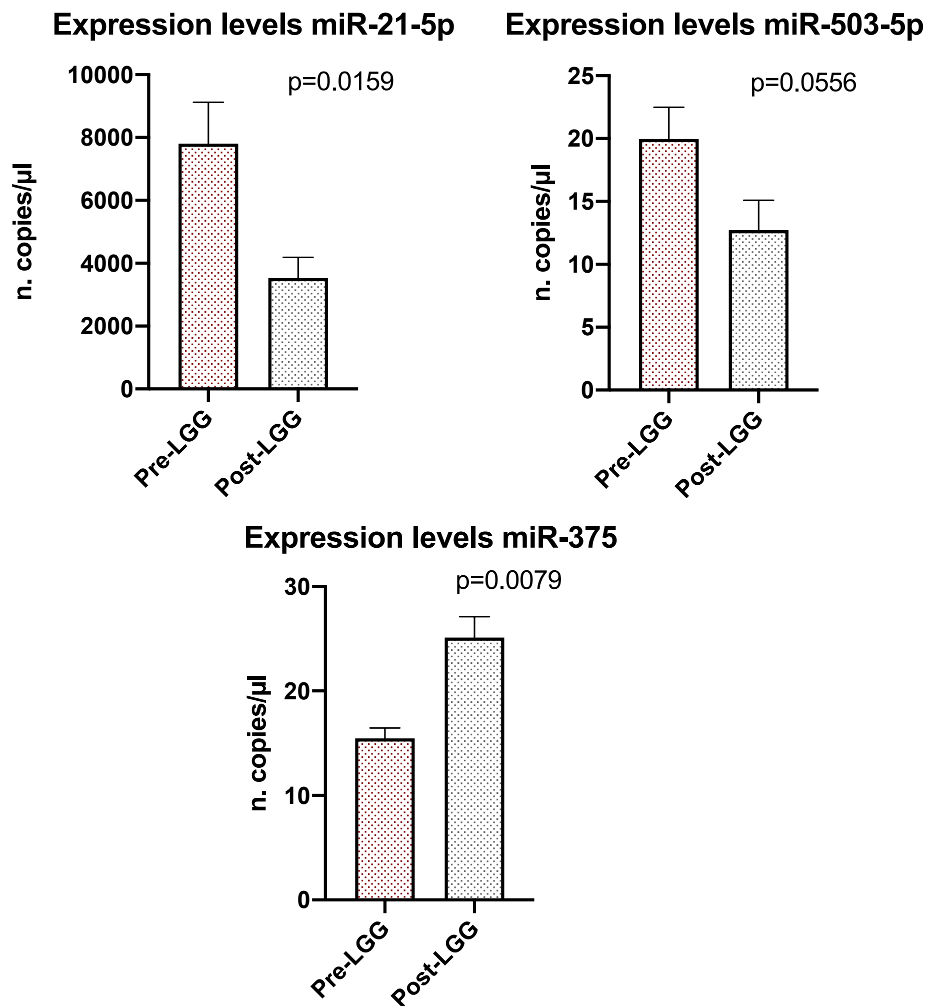

Figure 29. Change in expression levels of selected miRNAs before and after treatment with LGG. Mann-Whitney statistical test.

Ultimately, these data suggest that an integrated therapy based on the administration of probiotics is able to induce important epigenetic modifications in oncogenic and tumor suppressor miRNAs known to be involved in the development and progression of colorectal cancer.

All the data obtained in this study encourage the study of miRNAs as well as their use as diagnostic and prognostic biomarkers for CRC. In addition, the data obtained on the role of microbiota enrichment by probiotics suggest how integrated treatment may positively modulate the expression levels of miRNAs associated with the aggressiveness of CRC.

5. DISCUSSION

As widely described in the Introduction section, colorectal cancer (CRC) represents one of the most diagnosed and deadly tumor worldwide [Ferlay J. et al., 2019; World Health Organization, 2022]. Despite the advancement of both screening programs, diagnostic procedures and pharmacological treatments the management of CRC is still controversial and often tumors are diagnosed in a very advanced stage with a poor survival for patients. To face this current limitation in the early diagnosis of CRC and in the better management of this pathology, several studies have tried to identify novel effective biomarkers, however, confusing and conflictual results have been generated in this matter without the establishment of effective biomarkers translated into clinical practice [Lawler M. et al., 2018; Ogunwobi OO. et al., 2020].

More recently, a great attention was paid to the potential diagnostic and prognostic role of non-coding RNAs, and in particular miRNAs, i.e. small non coding RNA of 18-22 nt in length associated with several physiological and pathological processes. In this context, different computational and experimental investigations have demonstrated the effective diagnostic value of miRNAs in different tumors including oral cancer, breast cancer and uveal melanoma [Falzone L. et al., 2019; Crimi S. et al., 2020; Falzone L. et al., 2020], therefore, similar investigations were performed in CRC by using analytical workflows developed at the Laboratory of Experimental Oncology of the University of Catania.

More in detail, the use of miRNAs as non-invasive predictive biomarkers are sustained by their stability in different biological matrices and their easily analysis through molecular methods; therefore, miRNAs represent the perfect circulating biomarkers to analyze in liquid biopsy samples [Sayani M. et al., 2019].

Although the role of miRNAs in CRC was widely investigated by different researchers, no conclusive results have been obtained from these studies, therefore, the integrated analysis of bioinformatics data available on this topic together with clinical validation of the diagnostic potential of miRNAs may be helpful for the identification of reliable biomarkers for this pathology.

All these observations supported the investigations performed in the present study whose aim was to identify a panel of miRNAs with diagnostic and prognostic significance in CRC as well as to establish how microbiota and microbiome modifications may induce positive modulation of miRNAs associated with the development and progression of CRC.

For these purposes, a well-consolidated bioinformatics pipeline was adopted for the computational identification of miRNAs involved in CRC as previously demonstrated in other computational studies [Falzone L. et al., 2016; Hafsi S. et al., 2016]. While the validation experiments of miRNA expression levels in clinical samples were performed by using the high-sensitive ddPCR technologies suitable for the analysis of liquid biopsy samples [Crimi S. et al., 2020].

For these purposes, a preliminary bioinformatics investigation analysis was conducted to identify a panel of miRNAs potentially involved in the development and progression of CRC and thus potentially useful as diagnostic and prognostic biomarkers for this tumor.

Compared to other computational analyses reported in literature, innovative aspect of the present study was the execution of an integrated computational analysis performed taking into account all the relevant miRNA expression datasets contained in the GEO DataSets and TCGA databases. Overall, the computational investigations were performed on 13 miRNAs microarray expression datasets selected from the GEO DataSets database (only ten contained reliable miRNA expression data) and a miRNA expression dataset obtained from the TCGA PANCAN database selecting only data related to CRC and normal tissue.

Through these analyses, it was possible to identify a set of significantly dysregulated miRNAs by performing differential analyses between the expression levels of miRNAs in CRC samples compared to miRNAs in healthy tissues. The results obtained from the analysis of GEO DataSets miRNA expression data allowed the identification of 20 miRNAs significantly dysregulated in CRC. In particular, ten miRNAs were up-regulated and ten were down-regulated. One miRNA among the first category was subsequently

removed because of its nature as a tRNA fragment: the up-regulated hsa-miR-1308.

As regards the analysis of the miRNA expression dataset obtained from the TCGA PANCAN database, a huge number of dysregulated miRNAs in CRC was established (225 miRNAs of which 145 were down-regulated and 80 were up-regulated). By integrating these two analyses, it was possible to establish which miRNAs are involved in tumor development and progression. Among these, the most significant and up-regulated miRNAs were hsa-miR-21-5p and hsa-miR-503-5p while among the most down-regulated were hsa-miR-133b, hsa-miR-375 and hsa-miR-497-5p. The literature describes these miRNAs as frequently modulated and strongly involved in Colorectal Cancer. This notion further validates the consistency of the bioinformatics analyses conducted [Slattery ML. et al., 2016; Wei R. et al., 2017].

To better characterize the functional role of these miRNAs in CRC, further computational investigations were performed by using other bioinformatics tools including COSMIC and mirDIP. Through COSMIC, the most altered genes in CRC were identified while by using mirDIP it was possible to establish the miRNA-mRNA interaction between the selected miRNAs and the main genes altered in this tumor. These analyses revealed that the highest interaction levels with the selected genes were showed by hsa-miR-223-3p, hsa-miR-195-5p and hsa-miR-497-5p with the last two interacting almost with the same targets, noting that 9 out of 10 gene interactions were the same. It is worth to note that these 2 miRNAs are organized in a cluster on chromosome 17 p13.1 and this analysis also suggested the same functional roles [Flavin RJ. et al., 2009].

Of note, all these miRNAs are known to be involved in the pathogenesis of CRC. Indeed, hsa-miR-223 is recognized as a miRNA with a diagnostic value and involved in the progression of CRC [Ju H. et al., 2018; Chai B. et al., 2019]. Other studies have demonstrated that hsa-miR-497-5p and hsa-miR-195-5p are down-regulated in CRC [Qiu Y. et al., 2016; Zhang X. et al., 2016; Yang M. et al., 2017; Hong S. et al., 2019].

Then the targeted genes and pathways modulated by the selected miRNAs were identified by using the pathway prediction tool DIANA-mirPath. Through this

software, the main pathways altered by the selected miRNAs were identified emphasizing how these miRNAs were able to regulate key tumor-promoting signal transduction pathways including MAPK, p53, RAS and PI3K/Akt pathways. As regards the most targeted genes, DIANA-mirPath investigations highlighted a strong modulation of AKT, BCL2, CCND1, CCND2, CCND3, CDK4, CDK6, EGFR, MAPK family, VEGFA, etc., all genes known to play a fundamental role in CRC progression and development [Wee P. et al., 2017; Lin SH. et al., 2018; Ramesh P. et al., 2020].

Through all these computational investigations, it was possible to select four miRNAs potentially involved in the development and progression of CRC and useful as biomarkers for this pathology. In particular, two up-regulated and two-down-regulated miRNAs in CRC were selected for the validation analyses performed on FFPE and liquid biopsy samples obtained from CRC patients and healthy donors. These four miRNAs were hsa-miR-21-5p and hsa-miR-503-5p (up-regulated) and hsa-miR-375 and hsa-miR-497-5p (down-regulated).

The validation analyses to assess the diagnostic potential of the four selected miRNAs were performed by using the high-sensitive ddPCR amplification system with a protocol developed at the Experimental Oncology Laboratory of the University of Catania. The adoption of ddPCR was done as both FFPE and liquid biopsy samples are considered poor sources of miRNAs as FFPE samples contained very degraded miRNAs, while liquid biopsy samples may contain low amount of the investigated miRNAs.

By using ddPCR and analyzing the expression levels of CRC FFPE tissue samples versus normal adjacent mucosa it was possible to observe a significative increase in the expression levels of hsa-miR-21-5p and hsa-miR-503-5p and a significative decrease of hsa-miR-375 and hsa-miR-497-5p as predicted by the previous computational analyses. These results confirmed the predictive value of the computational investigation here performed and the bioinformatics pipeline developed by the Experimental Oncology Laboratory of the University of Catania.

Although the consistent results obtained through the analysis of miRNA expression levels in CRC samples versus normal mucosa, less robust results

were obtained when liquid biopsy samples were analyzed. Indeed, only hsa-miR-21-5p and hsa-miR-497-5p showed a significant increase and decrease of their expression levels in serum; while the other two miRNAs, hsa-miR-503-5p and hsa-miR-375 showed only a non-significant trend of increment and decrement, respectively.

The diagnostic potential of these miRNAs was further confirmed by performing ROC analyses which demonstrate that the four miRNAs in FFPE samples are reliable in diagnosing CRC compared to non-malignant lesions; while the analysis performed on liquid biopsy samples displayed only a limited diagnostic potential.

After validating the diagnostic potential of miRNAs, we were also interested in investigating their prognostic significance in the prediction of CRC patients' survival, however, no follow-up data were collected for the patients recruited in the study. Therefore, further computational analyses were performed to further establish the functional roles of the four validated miRNAs as well as their prognostic role in predicting patients' overall survival. For these purposes, miRTargetLink Human, STRING, GO Panther and OncoLnc analyses were performed.

Briefly, miRTargetLink Human analysis revealed that ten genes were concomitantly modulated by the four selected miRNAs while STRING and GO Panther analyses showed that these ten genes were able to interact with each other thus regulating different molecular and biological processes. Some of the ten identified genes are already known to be involved in CRC development and progression, such as BCL2 [Ramesh P. et al., 2020], ERBB2 and VEGFA [Cui W. et al., 2017; Ross JS. et al., 2018].

Subsequently, OncoLnc tool was used to better elucidate the prognostic potential of the four selected miRNAs in predicting patients' survival. The results obtained through this further tool revealed a good prognostic potential for circulating hsa-miR-375. In addition, the tissue levels of hsa-miR-21-5p, hsa-miR-503-5p and hsa-miR-375 can predict the overall survival rate of patients at the diagnosis.

As regards the second major endpoint of the study, significant data were also obtained about the modulating role of microbiota in the expression levels of miRNAs when enriched with specific probiotics.
In particular, the evaluation of the expression levels of the miRNAs hsa-miR-21-5p, hsa-miR-503-5p and hsa-miR-375 in a pilot series of patients affected by CRC and recruited within the two LGG clinical trials showed that treatment with the probiotic induces a significant reduction of up-regulated miRNAs and a significant increase of down-regulated miRNAs in the tumor. These important preliminary results obtained represent the basis for demonstrating how the enrichment of the intestinal microbiota in cancer patients can be useful not only as supportive therapy but also as a real treatment for the treatment of tumors.
These latter results are in line with the current literature according to which the enrichment of gut microbiota with probiotics induces several beneficial effects to the host both for the immune system but also from a molecular and epigenetic point of view [Torres-Maravilla E. et al., 2021; Zhou H. et al., 2021].
As regards the effects on miRNAs, it was demonstrated that gut microbiome actively interacts with host miRNAs and such interactions are responsible for the regulation of cellular and molecular processes involved in the development and progression of different tumors, including CRC [De Silva S. et al., 2021].
To the best of our knowledge, this is the first report on the beneficial effects induced by *Lactobacillus rhamnosus GG* on miRNA expression in CRC. A similar study has demonstrated the positive effects of another probiotic, *L. acidophilus*, which was associated with the improved expression of a lncRNA–miR–mRNA network useful for both monitoring purposes and therapeutic approaches [Khodaii Z. et al., 2022]. Therefore, this and other studies encourage the therapeutic application of probiotics on the modulation of miRNAs involved in the development of tumors [D'Amelio P. et al., 2017; Davoodvandi A. et al., 2021].

6. CONCLUSIONS

The results of the present study allowed the identification of a set of miRNAs significantly altered in colorectal cancer and potentially useful as diagnostic and prognostic biomarkers and to establish the beneficial effects of probiotics administration in the modulation of miRNAs associated with CRC.

These results were achieved by using both computational and experimental approaches to achieve more reliable results on the predictive potential of miRNAs and positive effects of microbial interventions in patients with CRC.

Specifically, through these analyses it was demonstrated the high diagnostic potential of the up-regulated miRNAs hsa-miR-21-5p and hsa-miR-503-5p and the down-regulated hsa-miR-375, while weaker results were obtained for hsa-miR-497-5p. Also the prognostic role of these miRNAs was confirmed by further computational evaluations, especially for hsa-miR-21-5p, hsa-miR-503-5p and hsa-miR-375.

Notably, better results were obtained in tissue samples, therefore, these miRNAs may serve as prognostic biomarkers to predict the survival of patients at diagnosis.

As regards the microbiota enrichment with LGG, the data obtained strongly encourage the implementation of supportive treatments to cancer patients based on the administration of probiotics able to modulate the expression levels of miRNAs responsible for a worse prognosis of patients.

Overall, the results here achieved represent a starting point for the implementation of further validation analyses performed on a wider cohort of patients. The effective limitations of this study are indeed the limited number of samples analyzed and the lack of clinical-pathological data of the patients enrolled in this study. Another limit is represented by the lack of samples and clinical data collected during the follow-up of patients. Despite these limitations, the computational and the experimental data here obtained support the use of the high-sensitive ddPCR amplification system as a reliable method for the evaluation of the expression levels of miRNAs in FFPE and liquid biopsy samples. In addition, the results obtained in CRC patients before and after the administration of CRC will clarify whether LGG can be suggested as an adjuvant

to be associated with anti-tumor treatment, especially in cases of colorectal cancer.

Finally, in the near future, the results here obtained will be used for the construction of a diagnostic workflow based on both epigenetic and microbial investigations to predict the aggressiveness of tumors and for the early diagnosis of suspicious lesions or in patients at risk for this tumor.

7. REFERENCES

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Appendix A

Supplementary Table 1. Dysregulated miRNA in CRC according to the data repoted in the TCGA PANCAN database

miRNA ID	p-value	Log2FC
Down-reguated miRNAs		
hsa-miR-328-3p	1.76364E-10	-6.105
hsa-miR-486-5p	2.13816E-12	-5.865
hsa-miR-139-5p	1.19006E-12	-5.710
hsa-miR-139-3p	6.26923E-13	-5.488
hsa-miR-129-5p	1.19761E-11	-5.208
hsa-miR-197-3p	2.10453E-12	-5.157
hsa-miR-149-5p	8.55049E-10	-4.965
hsa-miR-642a-5p	8.60869E-13	-4.837
hsa-miR-133a-3p	9.30528E-09	-4.819
hsa-miR-6511b-3p	2.84385E-10	-4.766
hsa-let-7d-3p	3.64711E-12	-4.720
hsa-miR-766-3p	1.81825E-10	-4.650
hsa-miR-125a-5p	6.46236E-13	-4.503
hsa-miR-150-5p	8.02153E-13	-4.456
hsa-miR-1306-5p	5.11433E-11	-4.447
hsa-miR-145-5p	1.09699E-08	-4.373
hsa-miR-574-3p	2.09534E-10	-4.254
hsa-miR-194-3p	2.10926E-11	-4.136
hsa-miR-378a-5p	1.42072E-21	-4.127
hsa-miR-497-5p	1.05076E-12	-4.017
hsa-miR-671-3p	1.09029E-15	-3.820
hsa-miR-1180-3p	2.45536E-13	-3.794
hsa-miR-193b-5p	3.60065E-09	-3.750
hsa-miR-375-3p	7.16822E-23	-3.695
hsa-let-7b-5p	1.02038E-21	-3.661
hsa-miR-92b-3p	2.22675E-11	-3.644
hsa-miR-99b-5p	8.22443E-12	-3.637
hsa-miR-193a-5p	6.09107E-10	-3.624
hsa-miR-296-5p	1.6688E-09	-3.547
hsa-miR-1296-5p	4.11949E-08	-3.540
hsa-miR-195-3p	8.42292E-12	-3.492
hsa-miR-150-3p	8.27789E-13	-3.471
hsa-miR-3605-3p	2.67128E-09	-3.465
hsa-miR-744-5p	8.45006E-14	-3.459

hsa-miR-423-3p	1.27995E-28	-3.436
hsa-miR-532-3p	2.12235E-14	-3.424
hsa-miR-146b-3p	9.21305E-11	-3.316
hsa-miR-181a-5p	3.75549E-10	-3.298
hsa-miR-125b-5p	8.90801E-13	-3.253
hsa-miR-629-3p	7.63142E-11	-3.249
hsa-miR-485-3p	9.30498E-09	-3.234
hsa-miR-193b-3p	3.04369E-11	-3.216
hsa-miR-23a-5p	1.38412E-07	-3.201
hsa-miR-1226-3p	1.14904E-09	-3.196
hsa-let-7b-3p	3.72236E-12	-3.185
hsa-let-7e-3p	3.31339E-09	-3.151
hsa-miR-324-3p	2.32968E-17	-3.151
hsa-miR-7702	1.32979E-06	-3.127
hsa-miR-326	2.75526E-06	-3.124
hsa-miR-3940-3p	1.49509E-09	-3.120
hsa-miR-3615	3.04119E-10	-3.078
hsa-miR-3917	6.68559E-08	-3.055
hsa-miR-1249-3p	9.09694E-08	-3.021
hsa-miR-433-3p	1.23338E-08	-2.976
hsa-miR-342-5p	3.24317E-10	-2.972
hsa-miR-4728-3p	2.25063E-08	-2.969
hsa-miR-3127-5p	5.67508E-10	-2.953
hsa-let-7c-5p	1.90162E-12	-2.951
hsa-miR-99b-3p	3.39598E-11	-2.943
hsa-miR-1307-3p	4.0898E-09	-2.926
hsa-miR-605-5p	2.65607E-07	-2.875
hsa-miR-3150b-3p	2.47965E-06	-2.836
hsa-miR-874-3p	1.31755E-17	-2.831
hsa-miR-29b-2-5p	9.7139E-12	-2.828
hsa-miR-484	4.81131E-12	-2.824
hsa-miR-378a-3p	1.02781E-19	-2.815
hsa-miR-1247-5p	4.14428E-08	-2.810
hsa-miR-370-3p	3.38904E-09	-2.759
hsa-miR-331-3p	3.49981E-11	-2.750
hsa-let-7i-3p	1.39857E-09	-2.697
hsa-miR-589-5p	9.73133E-17	-2.677
hsa-miR-339-5p	5.62071E-10	-2.645
hsa-miR-6720-3p	7.69338E-07	-2.643
hsa-miR-361-3p	4.05675E-10	-2.639

hsa-miR-6892-5p	5.34794E-08	-2.634
hsa-miR-30c-2-3p	2.26234E-11	-2.634
hsa-miR-191-3p	9.17368E-10	-2.617
hsa-miR-3173-5p	7.495E-10	-2.599
hsa-miR-15b-5p	3.37355E-11	-2.572
hsa-miR-3074-5p	9.5508E-10	-2.566
hsa-miR-3940-5p	1.14114E-07	-2.535
hsa-miR-3928-3p	8.36086E-08	-2.524
hsa-miR-1468-5p	1.74804E-14	-2.524
hsa-miR-7706	9.25601E-09	-2.516
hsa-miR-210-5p	4.60074E-10	-2.474
hsa-miR-483-3p	3.86932E-06	-2.425
hsa-miR-937-3p	1.81472E-09	-2.395
hsa-miR-133b	0.000128658	-2.364
hsa-miR-432-5p	7.93917E-08	-2.343
hsa-miR-455-3p	3.55241E-22	-2.274
hsa-miR-1343-3p	1.93886E-06	-2.264
hsa-miR-487a-5p	2.77973E-08	-2.254
hsa-miR-431-3p	2.43874E-08	-2.237
hsa-miR-4676-5p	1.51312E-08	-2.227
hsa-miR-2110	1.25422E-07	-2.171
hsa-miR-92a-3p	3.64681E-13	-2.148
hsa-miR-486-3p	4.31778E-05	-2.147
hsa-miR-5010-3p	2.98875E-08	-2.117
hsa-miR-128-1-5p	1.36797E-10	-2.108
hsa-miR-23b-5p	3.4577E-07	-2.097
hsa-miR-505-3p	2.91292E-09	-2.064
hsa-miR-214-3p	7.49572E-10	-2.060
hsa-miR-760	6.79437E-06	-2.051
hsa-miR-323a-3p	5.74519E-10	-2.043
hsa-miR-342-3p	6.22115E-09	-2.032
hsa-miR-6842-3p	3.08478E-06	-2.028
hsa-miR-2116-3p	2.4498E-07	-2.025
hsa-miR-125b-2-3p	5.44998E-07	-2.024
hsa-miR-130b-5p	1.65211E-09	-2.015
hsa-miR-363-3p	1.48512E-06	-1.997
hsa-miR-505-5p	2.38522E-08	-1.991
hsa-miR-1306-3p	8.04299E-09	-1.980
hsa-miR-501-3p	1.16757E-08	-1.978
hsa-miR-1271-5p	4.24167E-08	-1.966

hsa-miR-125a-3p	1.56021E-07	-1.956
hsa-miR-127-3p	4.38749E-18	-1.943
hsa-miR-3614-5p	1.99368E-06	-1.937
hsa-let-7a-2-3p	1.34572E-06	-1.889
hsa-miR-29c-5p	2.92115E-08	-1.887
hsa-miR-7704	0.000465702	-1.857
hsa-miR-324-5p	1.5005E-06	-1.854
hsa-miR-140-3p	3.09718E-10	-1.845
hsa-miR-675-3p	0.000337295	-1.833
hsa-miR-323b-3p	1.45679E-05	-1.810
hsa-miR-652-3p	6.49877E-07	-1.794
hsa-miR-132-3p	5.44246E-07	-1.753
hsa-miR-1228-3p	7.08324E-06	-1.750
hsa-miR-664b-3p	1.50854E-06	-1.741
hsa-miR-654-5p	1.24905E-05	-1.723
hsa-miR-423-5p	1.05253E-07	-1.703
hsa-miR-338-5p	4.50239E-05	-1.696
hsa-miR-1266-5p	6.43529E-06	-1.688
hsa-miR-5187-5p	3.45699E-06	-1.669
hsa-miR-4326	5.33087E-06	-1.667
hsa-miR-6716-3p	5.91254E-06	-1.653
hsa-miR-1229-3p	8.08064E-07	-1.652
hsa-miR-106b-3p	9.64955E-11	-1.646
hsa-miR-5698	0.000532159	-1.645
hsa-miR-891a-5p	7.6741E-05	-1.633
hsa-miR-377-5p	0.000412337	-1.617
hsa-miR-877-5p	0.000106625	-1.591
hsa-miR-330-3p	6.70362E-08	-1.577
hsa-miR-1301-3p	1.20691E-10	-1.570
hsa-miR-625-3p	1.40748E-08	-1.535
hsa-miR-1270	3.00526E-05	-1.526
Up-regulated miRNAs		
hsa-miR-598-3p	0.000362755	1.621
hsa-miR-27a-5p	3.57017E-05	1.643
hsa-miR-95-3p	1.45554E-05	1.644
hsa-miR-196b-5p	0.00020352	1.666
hsa-miR-146a-5p	0.000167128	1.684
hsa-miR-22-3p	6.20397E-08	1.685
hsa-miR-130b-3p	0.000151998	1.688
hsa-miR-26a-5p	3.57455E-07	1.691

hsa-let-7g-5p	3.75262E-05	1.691
hsa-miR-199a-3p	3.33112E-11	1.795
hsa-miR-199b-3p	3.84573E-11	1.797
hsa-miR-181d-5p	2.94286E-08	1.863
hsa-miR-34a-5p	2.47799E-05	1.906
hsa-miR-21-3p	5.8364E-09	1.990
hsa-miR-338-3p	5.39049E-08	1.993
hsa-miR-27a-3p	7.36536E-07	2.014
hsa-miR-10b-5p	1.70035E-05	2.034
hsa-miR-151a-3p	1.10169E-07	2.042
hsa-miR-451a	9.23912E-07	2.083
hsa-miR-22-5p	1.10834E-05	2.105
hsa-miR-337-3p	9.63823E-06	2.106
hsa-miR-224-5p	2.42466E-05	2.159
hsa-miR-146b-5p	5.15543E-06	2.159
hsa-miR-24-3p	1.62467E-08	2.191
hsa-miR-223-3p	1.23359E-06	2.225
hsa-miR-17-3p	6.25131E-08	2.299
hsa-miR-218-5p	4.97595E-08	2.345
hsa-miR-194-5p	5.55945E-05	2.365
hsa-miR-183-5p	1.42389E-09	2.488
hsa-miR-195-5p	2.52319E-08	2.513
hsa-miR-15b-3p	1.35006E-05	2.552
hsa-miR-192-5p	7.63008E-06	2.579
hsa-miR-196a-5p	4.74882E-07	2.613
hsa-miR-185-5p	2.75959E-10	2.638
hsa-miR-29c-3p	3.15443E-09	2.683
hsa-miR-127-5p	7.21967E-09	2.713
hsa-miR-7-1-3p	2.78813E-09	2.796
hsa-miR-30b-5p	2.33812E-07	2.852
hsa-miR-143-3p	3.79754E-07	2.856
hsa-miR-1307-5p	4.39251E-09	2.881
hsa-miR-10a-5p	2.38986E-09	2.966
hsa-miR-199b-5p	3.98407E-11	2.993
hsa-miR-2355-5p	3.74195E-10	3.052
hsa-miR-26b-5p	7.04045E-08	3.112
hsa-miR-152-3p	1.46391E-11	3.167
hsa-miR-30e-5p	2.83688E-19	3.198
hsa-miR-148a-3p	1.18267E-08	3.270
hsa-miR-126-3p	9.31928E-09	3.301

hsa-miR-200a-3p	3.7481E-09	3.355
hsa-miR-17-5p	3.34822E-10	3.382
hsa-miR-660-5p	1.28103E-08	3.384
hsa-miR-16-5p	2.82854E-09	3.538
hsa-miR-15a-5p	1.12383E-07	3.559
hsa-miR-552-3p	7.00634E-09	3.562
hsa-miR-203a-3p	6.64847E-10	3.712
hsa-miR-374b-5p	5.20982E-09	3.746
hsa-miR-98-5p	1.12467E-10	3.881
hsa-miR-582-3p	2.27502E-09	4.040
hsa-miR-379-5p	3.20908E-13	4.066
hsa-miR-144-5p	4.16874E-09	4.141
hsa-miR-215-5p	6.60074E-07	4.266
hsa-miR-126-5p	4.47343E-15	4.270
hsa-miR-335-3p	1.46365E-10	4.411
hsa-miR-182-5p	1.19514E-15	4.412
hsa-miR-503-5p	3.18901E-10	4.421
hsa-miR-203b-3p	2.89763E-14	4.519
hsa-miR-552-5p	1.00561E-09	4.546
hsa-miR-141-5p	1.14023E-13	4.555
hsa-miR-452-5p	9.59378E-11	4.569
hsa-miR-141-3p	1.60422E-14	4.570
hsa-miR-106b-5p	1.5724E-12	4.624
hsa-miR-101-3p	2.95592E-13	4.677
hsa-miR-20a-5p	2.95008E-11	4.920
hsa-miR-29b-3p	4.93081E-17	5.123
hsa-miR-429	1.05555E-10	5.134
hsa-miR-542-3p	1.202E-11	5.135
hsa-miR-142-3p	8.25533E-16	5.322
hsa-miR-19b-3p	2.13894E-13	5.556
hsa-miR-21-5p	7.90834E-12	6.221
hsa-miR-374a-3p	4.97028E-12	6.921