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Cancer stem cells biomarkers discovery for colorectal cancer: the role of miRNAs

Memoria presentada por **D. ANDREA PISANO**

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En Granada a 27 de enero de 2021

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“Chi dice che è impossibile non dovrebbe disturbare chi ce la sta facendo”.

“Los que dicen que es imposible no deberían molestar a los que lo están haciendo”

-Albert Einstein.

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1. RESUMEN

El cáncer colorrectal (CCR) es uno de los cánceres más comunes en el mundo. CCR permanece en silencio hasta veinte años y su detección temprana permite un buen pronóstico. Las células madre cancerígenas (CMCs), un pequeño grupo de células presentes en la masa tumoral, juegan un papel importante en el CCR, contribuyendo a un peor pronóstico, diseminación metastásica, recaídas y resistencia terapéutica. Ampliar las herramientas moleculares para comprender y detectar mejor las CMC podría mejorar la lucha contra el CCR. Para ello, un grupo de moléculas particularmente interesantes son los microARNs (miARNs), pequeñas secuencias (19-22 nt) de ARN con una función reguladora en organismos, cuyas alteraciones están implicadas en un gran número de patologías, incluyendo el CCR. Este contexto representa el tema del presente trabajo doctoral, que utiliza un enfoque multidisciplinar, enlazando modelos in silico, in vitro y ex vivo, para analizar y caracterizar los niveles de expresión de algunos miARNs involucrados en las vías de (desarrollo de) CMC y relacionados con el estadío de CCR. Con este fin, se utilizaron dos enfoques diferentes. En la primera fase del estudio, a través de una revisión bibliográfica en profundidad, se seleccionó un grupo de miARNs con un valor diagnóstico y pronóstico en el CCR. Los niveles de expresión de los miARNs seleccionados se analizaron en suero, tejido sano y tejido canceroso en un grupo restringido de pacientes con CCR. Dichos pacientes se dividieron en función de la presencia o ausencia de metástasis. Los miARNs seleccionados también fueron analizados en distintas líneas celulares: una línea celular sana usada como control (CCD-18Co), y tres líneas celulares de CCR (HCT-116, HT-29, T-84). Todas las líneas celulares se cultivaron siguiendo las condiciones estándar y se enriquecieron en CMCs, para reproducir in vitro las diferentes etapas de la patología. Según los resultados obtenidos, observamos una hiperexpresión de miR-21, miR-210, miR-34a, y una baja regulación de miR-16, la cual se asocia con el fenotipo de CMC. El miR-31b se expresó fuertemente en los tejidos tumorales y en el suero de los pacientes metastásicos, tanto en los cultivos en

monocapa como en los cultivos con CMCs. Se encontró que el miR-18a estaba regulado positivamente en todos los modelos tumorales, y se asoció con las CMCs y con el desarrollo de metástasis. Se halló que el miR-10b está regulado negativamente en los tejidos tumorales y en el suero de los pacientes no metastásicos. Nuestros resultados indican que los miARNs pueden ser utilizados como marcadores de CMC de CCR e incitan a profundizar en la investigación de las funciones de miR-21, miR-210, miR-10b, miR-31b y miR-18a. Al mismo tiempo, también se siguió una investigación de amplio espectro. Específicamente, el miRNoma de un grupo de 47 pacientes (controles sanos, pacientes con CCR sin metástasis y pacientes con CCR metastásico) se analizó mediante miARN-seq del suero, seguido de un real time RT-PCR. Esto, permitió la identificación de un comportamiento particularmente interesante de mir-486-5p, el cual mostró un pico de expresión en pacientes con CCR sin metástasis y una desaparición repentina en pacientes metastásicos, sugiriendo un papel protector contra la progresión de la enfermedad. Los niveles de expresión miR-486-5p fueron analizados mediante miARN-seq en las heces de un grupo diferente de pacientes en comparación con controles sano; mientras que en el suero y en los tejidos, además, se realizó un meta-nálisis de casos-control con los datos disponibles en la base de datos GEO. Los resultados mostraron la hiperexpresión de este miARN en las heces y en el suero de los pacientes; sin embargo, se observó un resultado opuesto en los tejidos. Para analizar más a fondo el comportamiento de este miARN, se llevaron a cabo ensayos con distintas líneas celulares, procedentes del tumor y enriquecidas en CMCs. El análisis mostró una disminución drástica en la expresión de miR-486-5p en el cultivo con CMCs. Tras estos resultados, se realizó un estudio funcional en la línea celular HCT-116, induciendo la expresión forzada del miR-486-5p o su inhibición. Posteriormente, se analizó el efecto sobre los factores de las CMCs y sobre las cuatro vías principales relacionadas con ellas, mostrando un efecto inhibitorio del miR-486-5p sobre el fenotipo de estas células. Estos resultados

proporcionan un paso adelante para comprender el papel del miR-486-5p en el CCR y en las CMCs, y, además, sugieren el poder de dicho miR para el diagnóstico y el pronóstico de CCR en combinación con otros biomarcadores de la enfermedad.

2. SUMMARY

Colorectal Cancer (CRC) is one of the most common cancer diseases in the world. This condition remains silent for up to twenty years and its early detection is linked to a good prognosis. Cancer stem cells (CSCs), a small group of cells present in the tumor mass, play an important role in the CRC, being linked to a worse prognosis, its metastatic dissemination, relapses and therapeutic resistance. Expanding molecular tools to better understand and detect CSCs could improve the arsenal in the fight against CRC. For this purpose, a group of molecules particularly interesting are the microRNAs (miRNAs), small sequences (19-22 nt) of RNA with a regulatory function in organisms, whose alterations are linked to a large number of pathologies, including CRC. This context represents the focus of the present doctoral work, which uses a multidisciplinary approach, involving in silico, in vitro and ex vivo models, to analyse and characterize the levels of expression of some miRNAs involved in the CSC pathways and in relation to CRC staging. To this aim, two different approaches were used. In the first phase of the study, through an in-depth literature revision, it was selected a set of miRNAs with a diagnostic and prognostic value in the CRC. The levels of these miRNAs were then analysed on serum, healthy tissue and cancerous tissue of a restricted group of CRC patients, divided in two groups by presence or absence of metastasis. The same miRNAs were then analysed on cell models: a healthy control represented by the cell line CCD-18Co, and three models of colon cancer: HCT-116, HT-29, T-84, cultured according to standard conditions and enriched in CSCs, in order to reproduce *in vitro* the various stages of the pathology. We observed an upregulation of miR-21, miR-210, miR-34a, and a downregulation of miR-16, associated with the CSC phenotype. miR-31b was strongly expressed in both monolayer and CSCs and tumor tissues and serum of metastatic patients. miR-18a was found to be upregulated in all tumor models and associated with CSC and metastatic condition. miR-10b was found to be downregulated in tumor tissues and serum of non-metastatic patients. Our results indicate that miRNAs can be used as

CSC markers of CRC and invite to deepen the roles of miR-21, miR-210, miR-10b, miR-31b and miR-18a. At the same time, a broad-spectrum investigation was followed; specifically, the miRNoma of a group of 47 patients (healthy controls, CRC patients without metastasis, metastatic CRC patients) was analysed by miRNA-seq analysis of the serum, followed by a real time RT-PCR analysis, allowing the identification of a particularly interesting behavior of mir-486-5p, which showed a peak of expression in patients with CRC without metastasis and a sudden disappearance in metastatic patients, suggesting a protective role against the progression of the disease. miR-486-5p expression levels were then analyzed by miRNA-seq in the feces of a different group of patients compared to healthy controls, while on serum and tissues a control case meta-analysis was performed from data available on GEO dataset. The results showed the hyperexpression of this miRNA in the feces and serum of the patients but offering an opposite result in the tissues. In order to further analyze the behavior of this miRNA, cell models have been produced, representative of the tumor and enriched in CSCs. The analysis showed a drastic decrease in the expression of miR-486-5p in the CSCs. Following these results, a functional study was carried out on the HCT-116 cell line, inducing a forced expression of miR-486-5p or its inhibition. Then, we analysed the effect on stem cell factors, and on the four main CSCs-related pathways, showing an inhibitory effect of miR-486-5p on the stemness phenotype of these cells: Theses results provide a step forward in understanding the role of miR-486-5p in CRC and CSC, and suggest its diagnostic and prognostic power in combination with other CRC biomarkers.

3. SINTESI

Il Cancro colon-retale (CCR) è uno dei tumori più diffusi al mondo. Questa condizione può rimanere silente sino a vent'anni dal suo esordio, inoltre, una sua tempestiva diagnosi è legata ad un buon outcome. Le cellule staminali del cancro, sono un piccolo gruppo di cellule presenti nella massa tumorale, che gioca un importante ruolo nel CCR, essendo connesse a una peggiore prognosi, alla sua disseminazione metastatica, a recidive e alla resistenza terapeutica. Aumentare gli strumenti molecolari, al fine di comprendere meglio e identificare le CSCs, consentirebbe di ampliare gli strumenti nella lotta contro il CCR. A tal fine, un interessante classe di molecole sono i microRNA (miRNAs), piccole sequenze di RNA (19-22 nt) con un ruolo regolatorio delle funzioni dell'organismo, alla quale alterazione sono legati un gran numero di patologie, tra le quali troviamo anche il CCR. Questo contesto rappresenta il focus della presente tesi di dottorato, la quale utilizza un approccio multidisciplinare, inclusi modelli in silico, in vitro ed ex vivo, per analizzare e caratterizzare i livelli di espressione di alcuni miRNA coinvolti nei pathways delle CSC e in relazione con gli stadi del CCR. A tale scopo sono stati utilizzati due approcci distinti. In una prima fase dello studio, a seguito di una approfondita analisi della letteratura, è stato selezionato un set di miRNA con un valore diagnostico e prognostico per il CCR. I livelli di questi miRNA sono stati analizzati nel siero, nel tessuto sano e in quello tumorale di un ristretto gruppo di pazienti affetti da CCR, questi sono stati divisi in due gruppi, in funzione della presenza o meno di metastasi. Gli stessi miRNA, sono poi stati analizzati su modelli cellulari: un controllo sano rappresentato dalla linea cellulare CCD-18Co e tre modelli rappresentativi di CCR, HCT-116, HT-29, T-84, coltivate secondo le condizioni standard ed arricchite in CSCs, al fine di riprodurre in vitro i vari stadi della patologia. Abbiamo osservato un iperespressione del miR-21, miR-210, miR-34 e una ipoespressione del miR-16, in associazione col fenotipo CSC. Il miR-31b è risultato essere iperespresso sia nelle colture tumorali coltivate secondo la metodologia classica che in quelle arricchite in CSCs, nei tessuti tumorali e nel siero dei pazienti metastatici.

Il miR-18a è risultato essere iperespresso in tutti i modelli tumorali e associato alle CSC e alla condizione metastatica. Il miR-10b è risultato essere downregolato nei tessuti tumorali e nel siero dei pazienti non metastatici. I nostri risultati indicano l'utilizzabilità dei miRNA come marcatori delle CSC del CCR, invitando inoltre uno studio più approfondito del ruolo di miR-21, miR-210, miR-10b, miR-31b e miR-18a. Allo stesso tempo, è stata eseguita un'analisi ad ampio spettro; nello specifico è stato analizzato il miRNoma nel siero di un gruppo di 47 pazienti (controlli sani, pazienti CCR non metastatici e pazienti CCR metastatici), tramite un approccio di miRNA-seq, seguito da un analisi tramite RT-PCR, permettendo l'identificazione di un comportamento particolare del miR-486-5p, che ha mostrato un picco di espressione nei pazienti CCR non metastatici, seguito da una significativa ipoespressione nei pazienti metastatici, suggerendo un ruolo protettivo di questo miRNA nella progressione del CCR. I livelli di espressione del miR-486-5p sono stati poi analizzati tramite miRNA-seq nelle feci di un altro gruppo di pazienti rispetto ai controlli sani, mentre su siero e tessuti è stata eseguita una meta-analisi caso di controllo da dati disponibili su dataset GEO. I risultati hanno mostrato una iperespressione del miR-486-5p nelle feci e nel siero dei pazienti, mentre è stato mostrato un comportamento opposto nei tessuti. Al fine di analizzare ulteriormente il comportamento di questo miRNA, la sua espressione è stata caratterizzata in modelli cellulari, rappresentativi del tumore e arricchiti in CSC. Queste analisi hanno mostrato una drastica ipoespressione del miR-486-5p nelle CSCs. A seguito di questi risultati, è stato condotto uno studio funzionale sulla linea cellulare HCT-116, inducendo un'espressione o inibizione forzata del miR-486-5p. Quindi, è stato valutato il suo effetto sui quattro principali pathways correlati al fenotipo CSC, mostrando l'effetto inibitorio del miR-486-5p su di esso: Questi risultati forniscono un passo avanti nella comprensione del ruolo del miR-486-5p nel CCR e nelle CSC, e suggeriscono il suo potere diagnostico e prognostico in combinazione con altri biomarcatori CCR.

4. INTRODUCTION

4.1.CANCER

4.1.1. Definition and origin

Neoplasms, divisible into benign and malignant, depending on their ability to spread into the organism, are also called tumours and in the case of malignant neoplasms, are defined as cancer. The eminent oncologist Willis described neoplasm as a mass of tissue whose growth exceeds if compared to normal tissues, continuing its growth also in absence of extern inputs (Sissons, 1953). The abnormal growth of these masses is due to two events, as first molecular alterations that occur in the cells, with consequent loss of cell cycle controls, as second the failure of the body mechanisms of control on altered cells (Hartwell & Kastan, 1994). The abnormal growth of the cells can occur in any part of the body and can drive to an alteration of the normal body functionality (Sudhakar, 2009). The WHO indicates cancer as a generic term that includes different kinds of diseases, united by the presence of abnormal cell growth and the possibility to spread into the body (Organization, 2018). Around the 5-10% of neoplasms are linked to inherited factors, while 90-95% of them are due to environmental factors, among the other: smoking habit, sedentarily, eating habits, infections, stress, ionizing irradiation, and pollution, making this a preventable pathology by changing lifestyle (Anand et al., 2008).

Cancer cells are characterized by some common aspects, such as: sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis, inflammation, and genomic instability and mutation (Hanahan & Weinberg, 2011) (Figure 1).

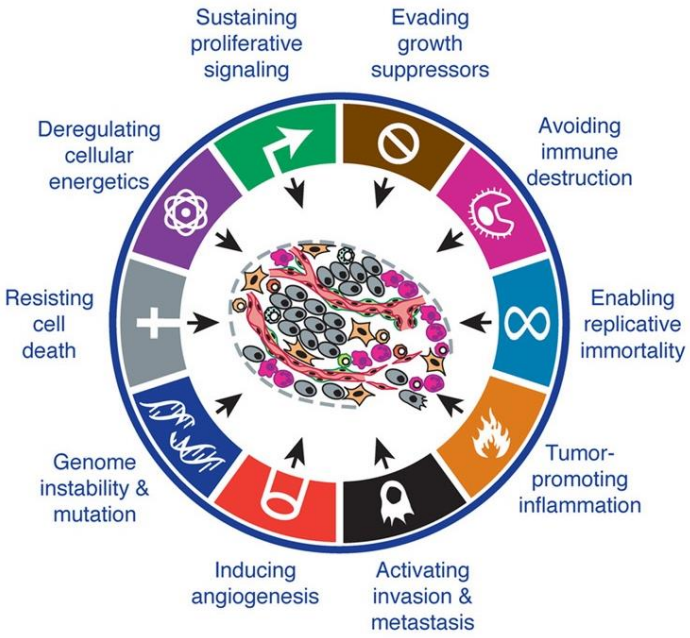


Figure 1. Cancer's hallmarks (Hanahan and Weinberg, 2011).

The neoplasm starts from the propagation of an altered single cell, that once avoided the control mechanisms starts a massive division, with the accumulation of genetic and epigenetic alteration, that contribute to the heterogeneity of the tumoral mass (Tammela & Sage, 2020) (Figure 2). Another important element that contributes to the heterogeneity of the tumour mass is the involvement of the non-cancer cells, with a fundamental role in the maintenance of tumoral mass. The tumoral microenvironment heterogeneity helps the tumor growth, from oxygenation to inflammation and metastasis (Balkwill et al., 2012; Marusyk & Polyak, 2010; Tammela & Sage, 2020).

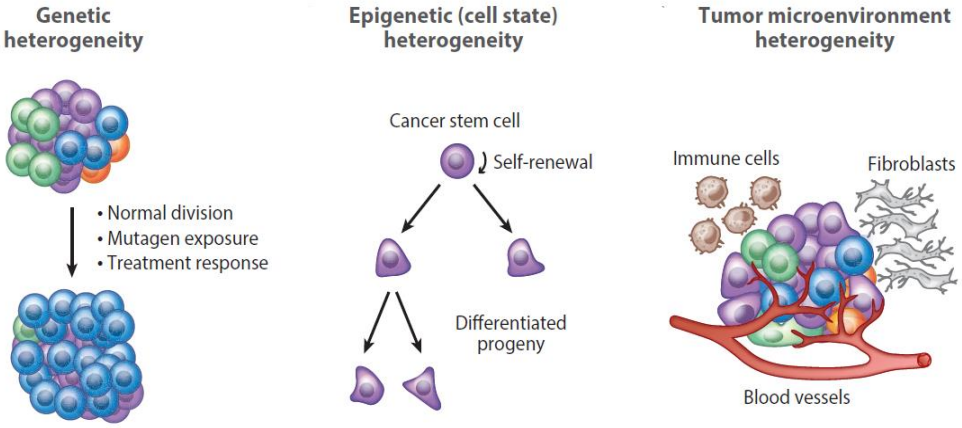


Figure 2. The three levels of tumor heterogeneity (Tammela and Sage 2020)

As mentioned before the conversion from a normal cell to neoplastic cell is driven by alteration in the DNA expression. These changes can occur in different classes of genes, such as proto-oncogenes, oncosuppressor, genes involved in programmed cellular death and those involved in DNA repair. These alterations can be used as makers for the tumor cell identification. An example of oncogene is *c-MYC*, which produces a transcription factor involved in cell growth, cell proliferation and cell metabolism. The translocation of these genes in more active sites of the DNA can induce hyperactivation of *c-MYC*, inducing uncontrolled cell growth and division (Boxer & Dang, 2001). While, an example of oncosuppressor alteration is the mutation of the sequence of *TP53*, the gene of p53, also known as the guardian of the genome, and the most muted gene in cancer (Berns, 2010). It is activated by DNA damages and induces cell cycle arrest and apoptosis (H. X. Yan et al., 2013). Considering the DNA damage repairing, we can take into consideration the mismatch repair (MMR). The alteration of this system is associated, among other, to genome-wide instability phenomena involved in a large number of cancers (G. M. Li, 2008).

4.1.2. Epidemiology

According to the WHO, cancer is the first cause of death in 48 countries and the second one in 43, in people before the 73 years (Organization, 2018) (Figure 3), with 70% of deaths from cancer in low- and middle-income countries (Organization, 2018).

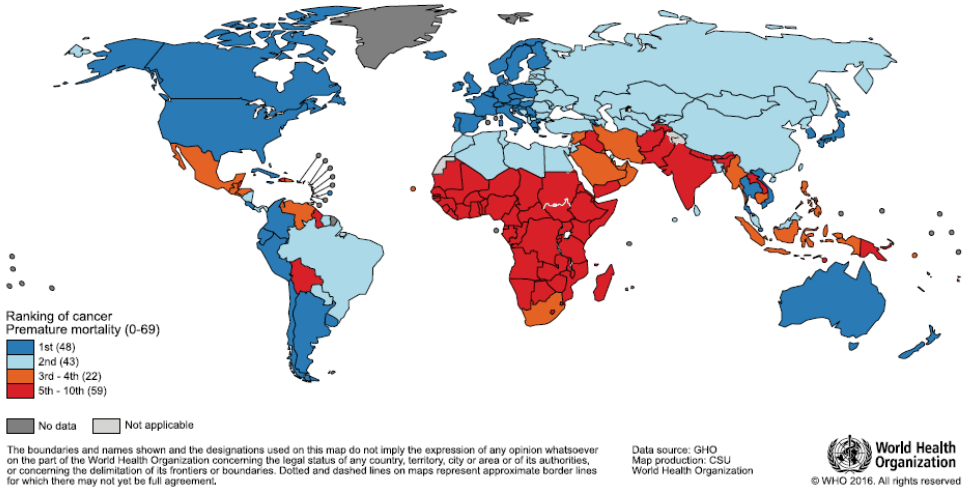


Figure 3. Global Map Presenting the National Ranking of Cancer as a Cause of Death at Ages Below 70 Years in 2015

The principal environmental causes of cell death are high body mass index, low fruit and vegetable intake, lack of physical activity, tobacco and alcohol abuse (Anand et al., 2008; Organization, 2018), and especially tobacco consumption causes 22% of death for cancer (Forouzanfar et al., 2015). The incidence and mortality of cancer are increasing in the last decades, where from 18.1 milion new cases, 9.6 milion deaths in 2018. Asia has the highest numbers of cases and deaths due to cancer, although it must be taken into account that there resides the 60% of the world population, followed by Europe, America, Africa and Oceania (Figure 4) (Bray et al., 2018).

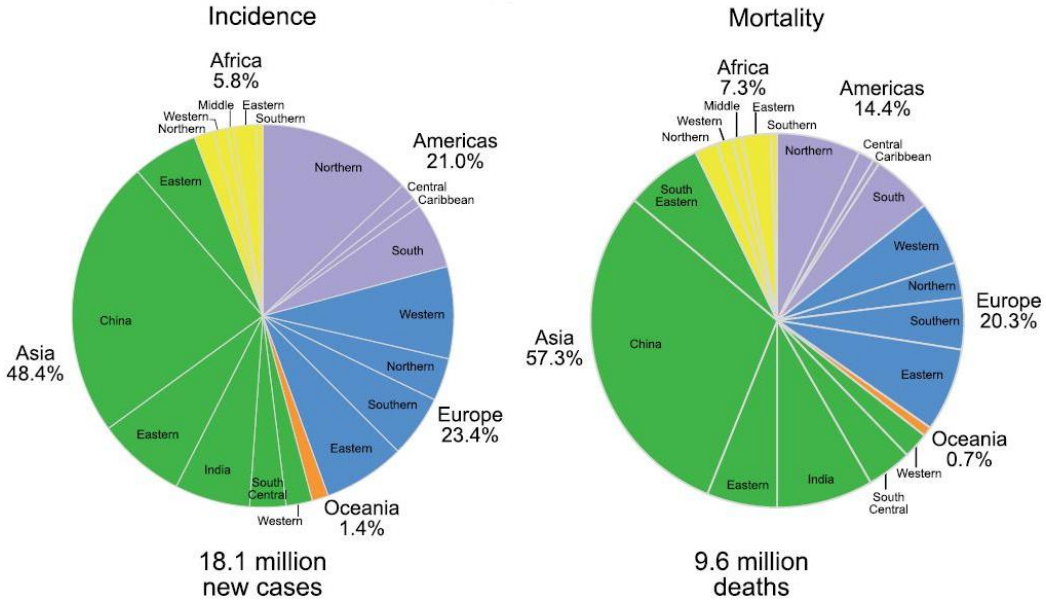


Figure 4. Data for cancer incidence and mortality. Source: GLOBOCAN 2018.

In both sexes, lung cancer is first cancer for incidence (11.6% of the total cases) and cause of cancer death (18.4% of the total cancer deaths), followed by breast cancer for incidence (11.6% of the total cases) and by CRC for mortality (9.2% of the total cases) (Figure 5).

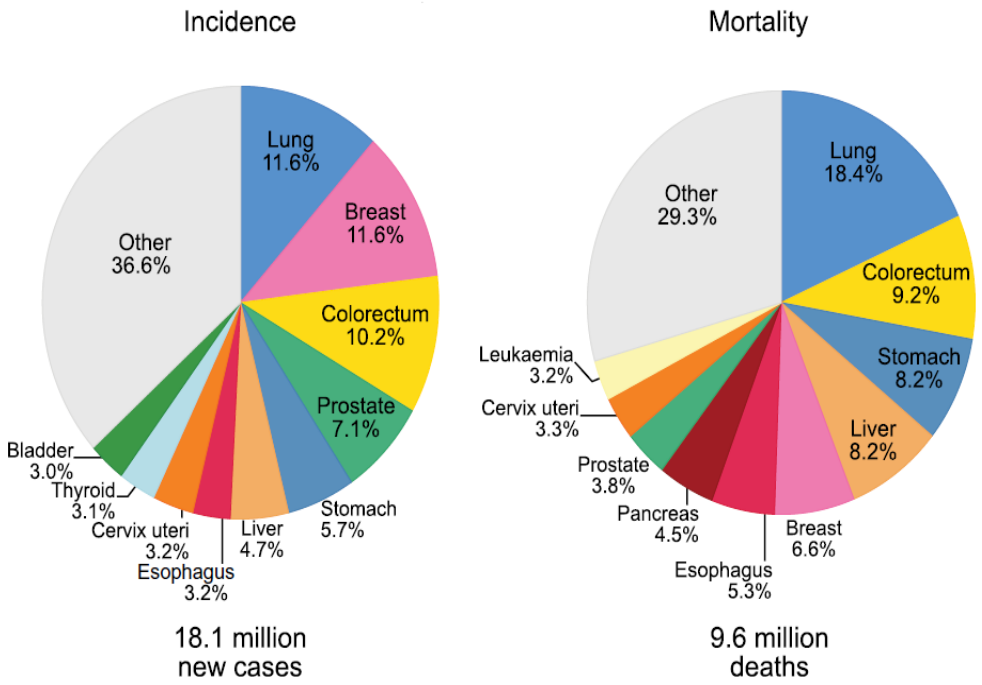


Figure 5. Pie chart present the distribution of cases and mortality for the 10 most common cancers in 2018 for both sexes. Source: GLOBOCAN 2018.

4.2.COLORECTAL CANCER

4.2.1. CRC epidemiology

Colorectal cancer is one of the main cancers for incidence and causes of death worldwide. Considering data for both sexes, CRC is the fourth cancer for incidence, with 6.1% of all cancer cases, after lung, breast and prostate cancers; while it causes 9.2% of death for cancer, second only to lung cancer for mortality (Figure 5).

In males, CRC represents third cancer for incidence with 10.9% of cases, after lung and prostate cancer, while it is the fourth cause of death for cancer, with 9.0%, after lung, liver and stomach cancer (Figure 6).

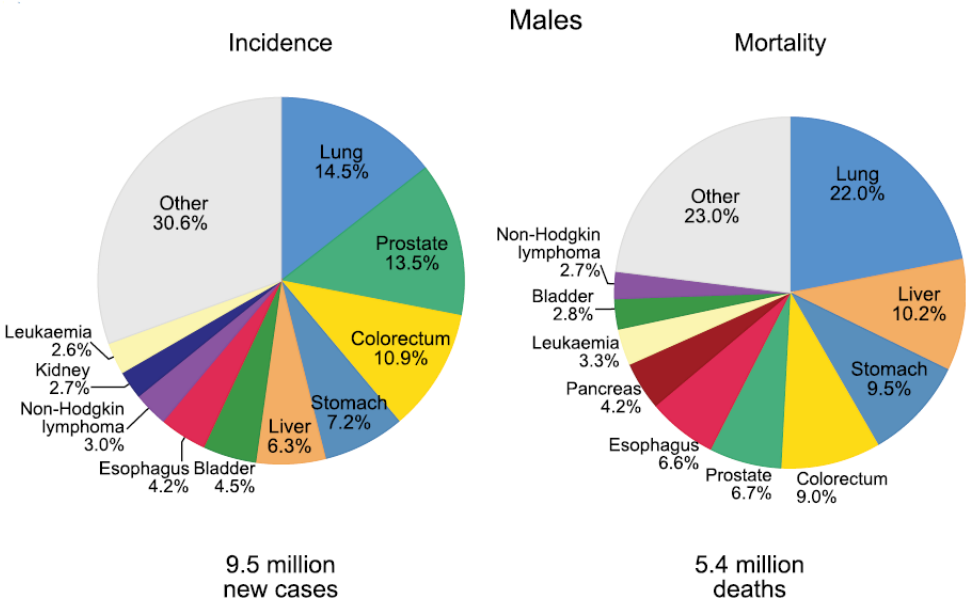


Figure 6. Pie Charts Present the Distribution of Cases and Deaths in males for the 10 Most Common Cancers in 2018. Source: GLOBOCAN 2018.

In females, CRC is second cancer for incidence, after breast, with 9.5% of new cases of cancer, while it is the third for mortality due to cancer, with 9.5% of death for cancer, after breast and lung cancer (Figure 7).

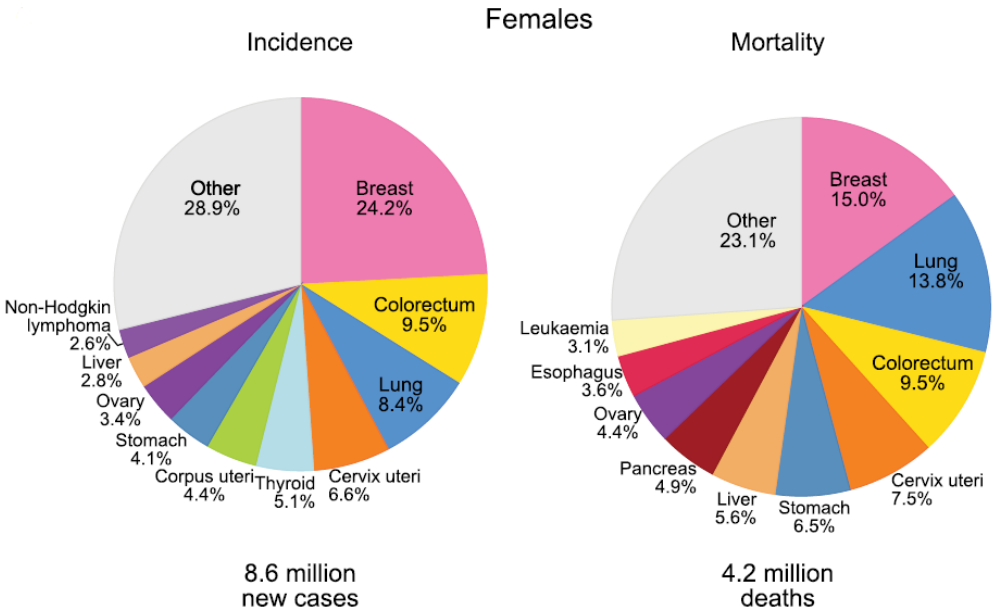


Figure 7. Pie Charts Present the Distribution of Cases and Deaths in females for the 10 Most Common Cancers in 2018. Source: GLOBOCAN 2018.

Countries with a high or very high human development index detent two-thirds of CRC cases (Figure 8) and about 60% of all deaths (Figure 9). Although in countries with high human development indices, such as the USA, Japan, Austria, and France, mortality and incidence rates of CRC are progressively decreasing, however, in countries with low development indices, such as the Philippines, China, Brazil, and others, the mortality and incidence rate of CRC are increasing. This tendency change might be due to the improvements in diagnostic procedures in the richest countries and to the westernization of the developing countries (Arnold et al., 2017).

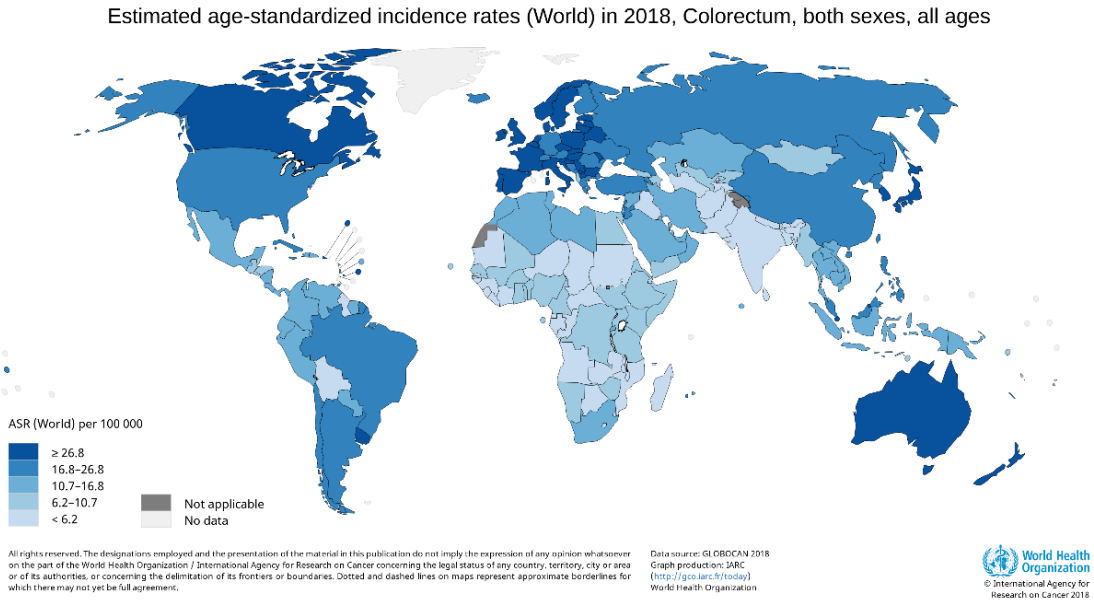


Figure 8. Worldwide colorectal cancer incidence rates (age adjusted according to the world standard population, per 100 000) in both the sex in 2018

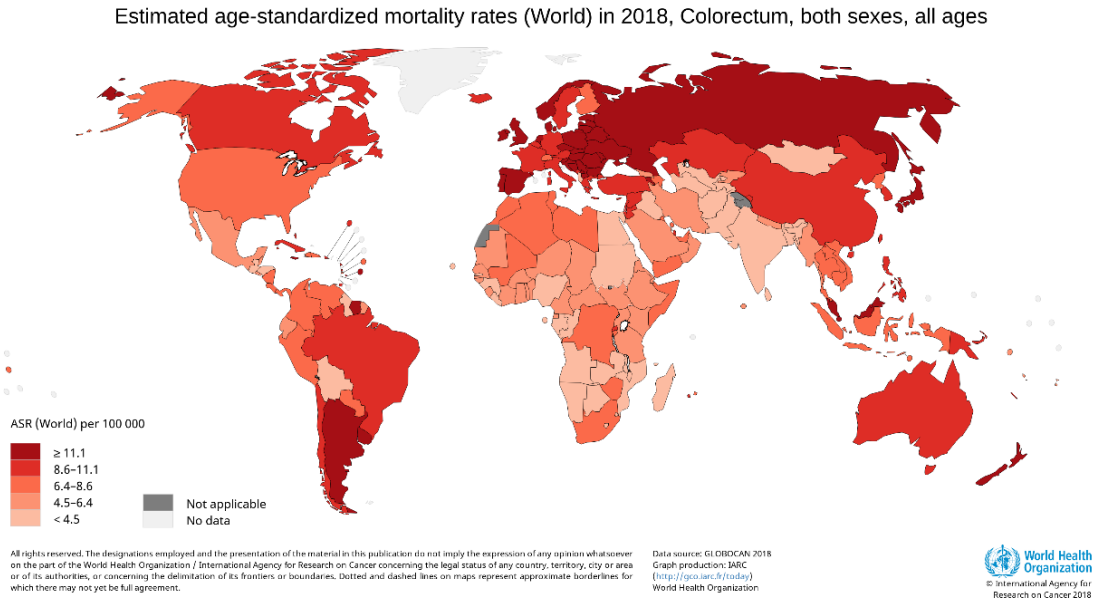


Figure 9. Worldwide colorectal cancer mortality rates (age adjusted according to the world standard population, per 100 000) in both the sex in 2018

4.2.2. CRC classification

Macroscopically, adenocarcinomas occur in different forms, as follow:

- **Vegetative**: it appears as a sessile neoformation that protrudes into the intestinal lumen, with a fungoid or "cauliflower" appearance, with an irregular surface and margins, which mainly affects the proximal colon.

- **Ulcerated**: malignant ulceration with a healthy bottom and raised, irregular, eversed margins; it can be circular or ovoid, with the largest diameter oriented along the transverse axis of the bowel in which it extends over 2/3 of the circumference.
- **Infiltrating**: it consists of central ulceration and a diffuse and extensive thickening of the wall, largely covered by normal mucosa; it often represents the evolution of a vegetative or ulcerated form and is frequently found in ulcerative colitis.
- **Annular-stenosing**: it extends for the entire intestinal circumference and causes a narrowing of the lumen; it has an irregular surface, with extensive necrotic processes and ulcerative tendency; it occurs mostly in the distal colon.

In the proximal colon, tract the CRC, presents itself as exophytic, sessile vegetative polypoid masses of fungoid appearance with irregular surfaces and margins, sometimes ulcerated and easily bled. In the distal colon tract, the tumor is annular-stenosing and tends to cause narrowing of the lumen with an obstructed channelling. In the rectal tract, this neoplasia has a vegetative or ulcerative appearance, which tends to bleed easily.

On the basis of the histological derivation, the CRCs can be divided into non-epithelial and epithelial. The non-epithelial derived tumours include: lipomas, leiomyomas, leiomyosarcomas, GISTs, angiosarcomas and malignant lymphomas. The derived epithelial colorectal tumours include: adenoma, dysplasia related to chronic inflammatory diseases (low and high grade intraepithelial glandular neoplasia) and carcinomas (Dionigi, 2011). Adenomas represent benign tumours, and can be the precursors of the malignancy. Adenomas are divided in polypoid and non-polypoid. The polyps can be tubular, villous, tubular-villous and serrated. The non-polyposid forms include ulcerative or infiltrative lesions. Carcinomas are malignant tumours, and include: adenocarcinoma, mucinous adenocarcinoma, signet ring adenocarcinoma, small cell adenocarcinoma, adenosquamous carcinoma, medullary carcinoma, undifferentiated carcinoma (Dionigi,

2011). Among the CRC, adenocarcinoma is the main occurring type of cancer, representing 90% of all the CRC (Fleming et al., 2012). According to histological analysis, the WHO identifies a grading of colorectal neoplasms in which three levels of malignancy are reported based on the degree of differentiation (Dionigi, 2011) (Figure 10):

- **Grade I:** in which the tubular-glandular organization appears well differentiated, with poor nuclear pleomorphism and few mitoses.
- **Grade II:** the glandular structure appears preserved; the cells often appear disordered in one or more layers and with numerous mitoses.
- **Grade III:** subversion of the primitive organization, irregular clusters of cells are detected, with numerous mitoses.

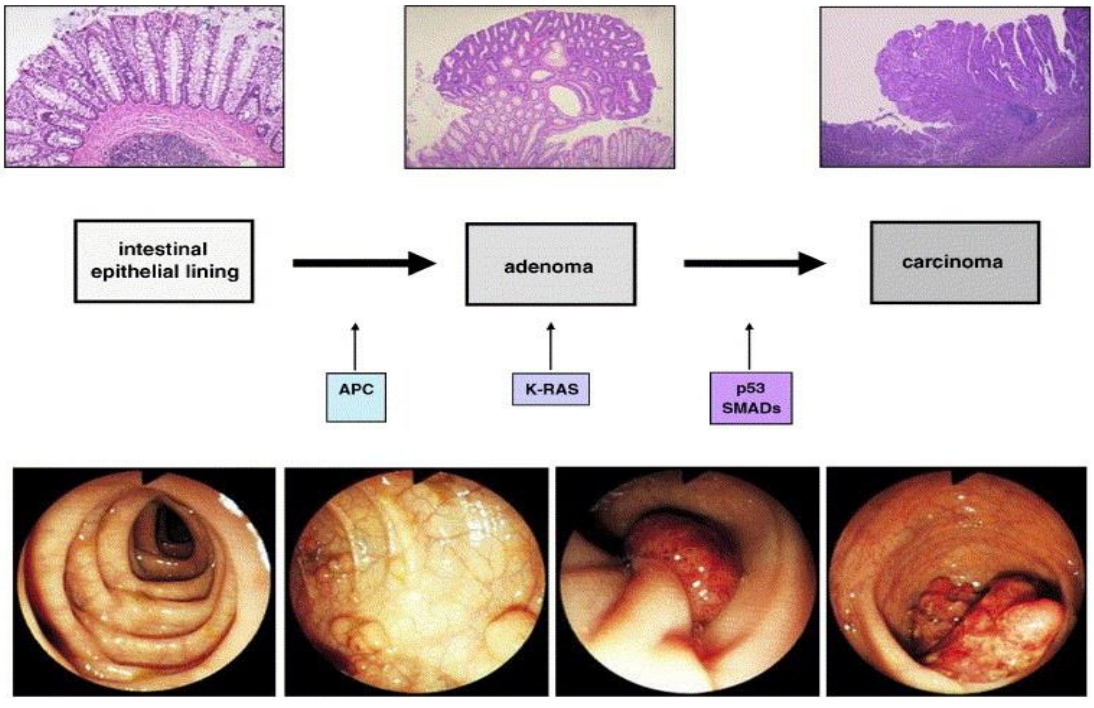


Figure 10. Sequential changes leading to the evolution of the CRC

4.2.3. Risk factors

The risk factors of CRC are environmental or inherited, but also modifiable and no modifiable. CRCs can occur in three ways: sporadic inherited and familial.

4.2.3.1. Environmental risk factors

CRC is widely considered an environmental disease, associated with a wide range of often ill-defined factors, such as cultural, social, and lifestyle. In fact, several studies show the increment of the incidence rates in migrants from low-risk to high-risk countries (Janout & Kollárová, 2001; I. T. Johnson & Lund, 2007; Mousavi et al., 2012). Body mass index (BMI), low physical activity, poor diet (high levels of the red and processed meat, low intake of fibers, whole grain and calcium), smoking cigarettes, heavy alcohol consumption (C. M. Johnson et al., 2013; Keum & Giovannucci, 2019) are remarkable risk factors. Obesity is accompanied by inflammation and insulin resistance (Ouchi et al., 2011), inflammatory conditions in the tumour microenvironment promote tumour growth and progression in CRC (Park et al., 2014). Furthermore, it has been demonstrated that an increment of 10 cm in the circumference increase of the 4% the risk to develop of CRC (Keum & Giovannucci, 2019). BMI is influenced by alimentary habits and physical activity. It has been demonstrated that modifying alimentary habits may reduce the risk of CRC up to the 70% (Willett, 2005), and physical activity reduce CRC risk through inducing gut motility, activating the immune system, and reducing inflammation and metabolic hormones (Ruiz-Casado et al., 2017). Alcohol, with its primary metabolite acetaldehyde, is classified as carcinogenic for human by the International Agency for Research (International Agency for Research on Cancer, 2012), and it is able to induce direct DNA damages and altering its proper synthesis and methylation (Seitz & Stickel, 2007). Cigarette smoke, in the function of its high content of toxic components, is able to induce irreversible colorectal mucosae damage, both by direct effect, by ingestion and an indirect effect through the circulatory system. It is able to induce genetic and epigenetic alterations, otherwise, it needs a long time before to induce carcinogenic effects (Limsui et al., 2010).

4.2.3.2. Unchangeable risk factors

Colorectal cancer occurs, in 90% of the cases, after age 50 years, with the peak of diagnosis between age 65 and 79 years (GLOBOCAN 2012 (IARC), 2016) (Figure 11). The effect of the age in the insurgence and death for CRC is more evident in males (Figure 10) (GLOBOCAN 2012 (IARC), 2016; Keum & Giovannucci, 2019).

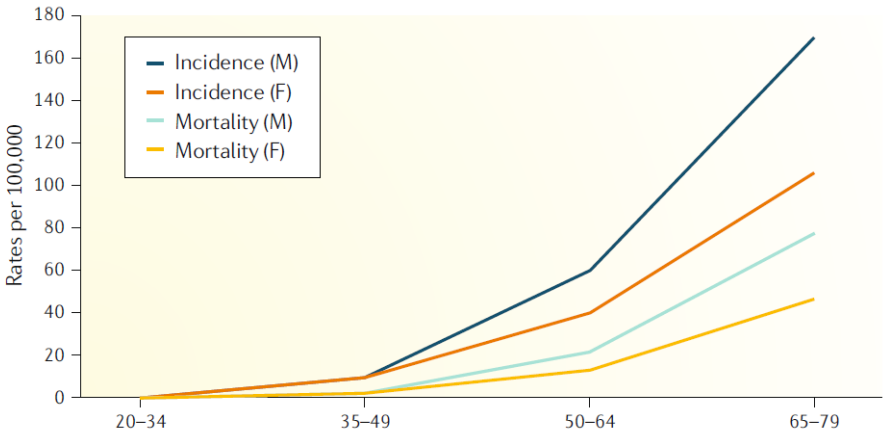


Figure 11. Incidence and mortality of colorectal cancer by age and sex worldwide.

Another risk factor is related to the familial history of CRC. Nonetheless, the ¾ of cases of cancer is sporadic, with negative familial history. In case of one 50-70 years first-degree relatives with CRC diagnosis, the risk of CRC incidence is double, and triplicated if the age of the relative is under the age 50 years or in case of two or more first-degree relatives with CRC diagnosis (Kuipers et al., 2015). A meta-analysis of observational studies evidenced that the risk to develop CRC is 2.24 higher in subjects with a first-degree relative affected by CRC, and rise up to 3.97 in case of two or more first-degree relatives (Butterworth et al., 2006). When CRC diagnosis occurs before age 50 years, it is often associated with a hereditary predisposition (Pearlman et al., 2017). Among the latter are included polypoid and non-polypoid forms. The first group includes: familial adenomatous polyposis (FAP), polyposis associated with MUTYH (MAP) and hamartomatous polyposis syndromes (e.g. Peutz-Jeghers, juvenile polyposis, Cowden syndrome); while among hereditary diseases without polyposis there is hereditary cancer

of the rectum non-polyposic colon (HNPCC is also known as Lynch syndrome). These conditions are all associated with a high risk of developing CRC and, in many cases, genetic mutation has been identified.

- Lynch syndrome (LS) is the most common of all hereditary cancer syndromes. They are implicated as the cause of LS, DNA mismatch repair gene mutations: *MLH1*, *MSH2*, *MSH6* and *PMS2*, which are the genes that produce MMR proteins (Tiwari et al., 2016).
- The second most common inherited syndrome that leads to the onset of CRC is familial adenomatous polyposis. This syndrome is caused by mutations in the *APC* (adenomatous polyposis coli) gene, which controls the activity of the Wnt signalling pathway (Vasen et al., 2015).

4.2.4. Natural history and molecular biology of CRC

In general, CRCs' natural history through four phases: initiation, in which DNA damage activates the neoplastic activation. After initiation starts the propagation phase in which there is an abnormal proliferation; other genetic and epigenetic transformations confers to the tumour cells an advantage in respect to the other cells. It is here that the tumour cells acquire a malignancy, also named as progression; where tumor cells can metastasize, living the primary sites of the tumor and spreading in the body (Pitot, 1993). The succession of these phases is faster in hereditary tumours. Furthermore, CSCs seem to improve cancerogenesis, by dividing rapidly and continuously and thus forming a proliferative cancer cell population (Munro et al., 2018). There are three mechanisms on the base of the CRC carcinogenesis: chromosomal instability (CIN), CpG island methylator phenotype (CIMP) and MSI (Grady & Carethers, 2008; Ogino & Goel, 2008).

CIN affects the structure and the number of chromosomal copy number, caused by errors during mitosis (Grady & Carethers, 2008). It occurs in 80-85% of CRCs, and follows the

adenoma-carcinoma sequence, in which specific genetic events correlate with the morphological evolution of the tissue in the progression from adenoma to CRC, in a multistep process. Characteristic mutations of this model are present in tumor suppressor genes, such as *APC*, *TP53*, and *DCC*, and in oncogenes, such as *KRAS* (Dow et al., 2015; Fearon & Vogelstein, 1990). The first precocious event is the polyps proliferation in the gout lumen, this event occurs in consequence of the *APC* inactivation, followed by the hyper activation of the Wnt/β-catenin signalling pathway, causing cell proliferation and adenoma volume increment (Dow et al., 2015). Wnt is a ligand that, by the nucleus translocation of β-catenin and its binds with TCF/LEF, induces the expression of some genes that regulate the cell cycle, such as cycline D and *c-MYC*, and genes related to cell progression *MMP-7* and *MMP-26*. In normal conditions, in absence of Wnt, APC with axin and GSK3β forms a complex that bind and induces the destruction of β-catenin. Furthermore, APC intervenes in other cellular processes, independent to Wnt, such as cytoskeleton regulation in the cell division, in the chromosomal alignment and in cell adhesion (Morán, 2010). The second event that maintains the adenoma evolution is on the charge of the oncogene *KRAS*. Recently, it has been shown that the mutant KRAS promotes hyperplastic growth in the epithelium of the colon (through stimulation of *MEK*) and suppresses differentiation in colon carcinomas with mutated APC. It also regulates cell polarity. Therefore, during the development of CRC, epithelial cells can lose their polarity and adhering junctions that mediate cell-cell contacts (Morán, 2010). Deletion of *DCC* and *SMAD 2-4*, implicated in the TGF-β pathway induces the tardive adenoma formation (Jass, 2006). The successive inactivation of *TP53* tumour suppressor gene contributes to the progression to CRC (Grady & Carethers, 2008). Other genes involved in this process are: *CTNNB1*, *BRAF*, *TGFBR2*, *PIK3CA*, *ARID1A*, *SRY*, *SOX9*, *FAM123B* and *ERBB*, involved in different pathways, such as Wnt/β-catenin, *EGF*, *MAPK*, *PI3K* and *TGF-β* (Morán, 2010).

Another important mechanism that characterizes the CRC insurgence and characteristic of Lynch syndrome is the MSI, which occurs in 15-20% of sporadic CRC. Microsatellites are short sequences repeated in tandem throughout the genome. An accumulation of frameshift mutations is caused by a primary defect in mismatch repair genes (MMR). In fact these genes, in normal conditions, repair the errors made by the DNA polymerase in the genome. The most affected genes of this mechanism are *hMLH1* and *hMSH2*. Genes that suffer the effect of this phenomena are: *TGFβRII*, *IGF2R*, *BAX*, *MSH*, *MSH6*, *caspase 5*, *APC*, *β-catenin*, *TCF-4*, *axine*, *MMP-3*, *E2F-4*, *BCL-10*, *CDX-2* and *hRAD50* (Morán, 2010).

The third mechanism involved in CRC is the CpG island methylator phenotype (CIMP), an epigenetic phenomenon that occurs in the promoter regions of tumour suppressor genes (such as *MLH1*, *MINT1*, *MINT2* and *MINT3*) with consequent silencing of expression (Markowitz & Bertagnolli, 2009b; Mojarad et al., 2013). There are two forms of CIMP: *CIMP1* and *CIMP2*. *CIMP1* is linked with MIS and mutation on *B-RAF*, while *CIMP2* is linked with *KRAS* alterations, or with the less common inactivation on B-RAF or p53 (Puccini et al., 2017). Other genes undergoing hypermethylation are: *RSSF1A*, an oncosuppressor that act in the G1/S phase of the cell cycle, inducing its rest; E-Cadherin, which mediates the cell adhesion, maintaining the tissue structure, promotes cell invasion and spread; and the gene codifying for *p16INK4a*, that inhibits CDK4 and 6 and prevent the phosphorylation of RB causing rest of the cell cycle in phase G1(Miranda et al., 2006).

Another important protagonist in the onset of CRC is EGFR. This receptor is bound, among others by EGF and TGFα. The bond triggers a series of events that lead to the activation of several pathways, including the RAS-RAF-MAPKs cascade and the PI3K/AKT pathway. Its hyperexpression or hyperactivation can lead to the development of CRC, due to an effect on cell growth and apoptosis (Normanno et al., 2006).

4.2.5. Clinical features

The precocious phases of CRC, in the majority of the cases, are silent and symptomatology appears in the advanced phases, with typical manifestation, in the function of the site affected by cancer. Furthermore, the paraneoplastic lesions bleed just in the 5% of the cases (Rugarli, 2010). The first symptoms are: localized or diffuse greasing abdominal pain, with in some cases borborygmi and abdominal distension, constipation and intestinal blockage, due to obstruction of intestinal transit by neoplastic mass. Then, bleeding is observed, which tends to increase as the disease progresses (Rugarli, 2010). When the tumours occur in the right colon, the content is prevalently liquid, the lesions that develop at this level are typically vegetative, ulcerated and easily bleeding. Therefore, the main symptoms will be it is referred to a chronic blood drip, with blood mixing with the intestinal content and is difficult to identify. The pain is intermittent and not very intense, localized in the right abdominal quadrants, sometimes associated with dyspeptic symptoms and asthenia due to anemia. In advanced forms a mass can be detected in the right quadrants. Anorexia and slimming are indicators of advanced stages of the pathology. When CRC occurs in the left part of the colon, it is characterize by constipation and diarrhea, with presence of blood and mucus in faces. This condition occurs because the material that passes through this tract is predominantly solid, this part of the intestine has a smaller diameter and the tumours of these seats are, for the most part, of an infiltrating annular type. The pain, often intermittent, occurs in the left abdominal quadrants due to the attempts of the colon to proceed the fecal material through a lumen restricted by the lesion. Continuous pain may be an expression of infiltration of the parietal peritoneum. Rectal tumours that affect the sigmoid colon present the same symptomatology of the left colon. In the ampullary region of the rectum, tumors are largest and present vegetative and ulcerative lesions. It is characterized by tenesmus and burdensome pain, sometimes associated with defecation which also determines rectory,

but which can occur independently of it. Perianal and perineal pain is detected in the sub-ampoules, which is accentuated during defecation, especially in rapidly stenosant forms. At times it is evident emission of nastriform feces mixed with blood and mucus with important darkness (Dionigi, 2011; Rugarli, 2010).

4.2.6. Diagnosis and prevention

In order to guarantee a good prognosis a precocious diagnosis is essential, in this regard a good prevention and screening campaign is desirable (Valori et al., 2012).

The occult blood test is a non-invasive test and consists in the research of blood in stool on three consecutive defections. It is recommended every two years in the population aged between 50 and 69. It is one of the principal screening methods and guarantees a large surveillance of the population. Various techniques are used in CRC screening, including faecal immunochemical testing, high-sensitivity guaiac-based faecal occult blood testing, multi-target stool DNA-testing, colonoscopy, computed tomographic colonography, and flexible sigmoidoscopy (Smith et al., 2019). Pancolonoscopy is considered as the gold standard method for the diagnosis. The colonoscopy is often not well accepted from the patients. It has a sensitivity of 96-97% and an accuracy of 90%. The risk of perforation in of the 0.1% and the risk of death is estimated between 0.01 and 0.03%. During pancolonoscopy is possible to do biopsies in order to take an intestinal sample for histological analyses (Dionigi, 2011; Grossi, 2019). A less invasive technique is the rectosigmoidoscopy, that consists in the analysis of the last 60 cm of the digestive tract, rectum, sigma and colon. It can be useful for the identification and removal of polyps in these areas. In case of positivity requires the use of pancolonoscopy (30% of the cases). To ensure greater sensitivity of the analysis, it can be associated with virtual colonoscopy and double-contrast clisma. The latter technique consists in the administration of a double-contrast clisma of air and barium, followed by an X-ray. With

the double-contrast clisma it is possible to identify typical pictures in case of vegetative lesions, which appear as marginal lesions and stenosis, with the typical apple core appearance. Other examinations such as ultrasound, CT and MRI scan allow the identification of secondary locations of the tumor (Dionigi, 2011; Grossi, 2019).

The prognosis and the treatment choice is determined by the TNM classification, carcinoembryonic antigen (CEA) and Ca 19.9 quantification, evaluation of microsatellite instability, and mutations in *KRAS* (KRAS proto-oncogene, GTPase), *NRAS* (NRAS proto-oncogene, GTPase), *BRAF* (v-RAF murine sarcoma viral oncogene homolog B), and mismatch repair (MMR) genes (Grossi, 2019). However, the available diagnostic and prognostic methods present some limitations. Some of these procedures are invasive and require the tolerance and adequate patient preparation (Walter et al., 2019); or in some cases these are less sensitive (Grossi, 2019). These facts made it necessary to advance in the field of diagnosis and screening for CRC. Recent evidences indicate microRNAs (miRNAs) as good candidate markers for diagnostic and prognostic procedures in cancer and in CRC (Imaoka et al., 2016).

As mentioned before the TNM classification is useful for both prognostic evaluation and treatment choice. The TNM classification allows to determinate the stage of the CRC. In this classification T indicate the level of tumor penetration, it is divided in four levels: Tx not assessable primitive neoplasia, T0 no evidence of primitive neoplasia, Tis intraepithelial carcinoma in situ or invasion of the lamina propria, T1 neoplasia confined in the submucosa, T2 neoplasia that invades the muscularis propria, without going beyond it, T3 neoplasia that passes the muscularis propria, with invasion of the dangerous tissues, T4a neoplasia infiltrating the visceral peritoneum, T4b neoplasia with direct invasion or adhesion to adjacent organs/structures. N indicates the lymph node invasion, it is divided in: NX lymph node cannot be assessed, N0 no regional lymph metastases, N1 metastases in 1-3 regional lymph nodes, N1a metastases in 1 regional lymph node, N1b metastases

in 2-3 regional lymph nodes, N1c satellite tumour deposits in the serosa or non-peritonealised pericolic and perirectal tissues without evidence of regional lymph node metastases, N2 metastases in 4 or more lymph notes, N2a metastases in 4-6 lymph node, N2b metastases in 7 or more lymph nodes. M indicates distant metastases, Mx indicate no evidence of distant metastases, M1 indicate distant metastases, M1a one organ limited metastases (liver, lung, ovary, not regional lymph nodes), M1b metastases in more than or in the peritoneum. The TNM classification is used to individuate the stage, as indicate in the following table (I. et al., 2015).

Table 1 CRC staging classification according to the TNM classification. Source(I. et al., 2015)

TNM Stage	Primary Tumour	Lymph Metastasis	Distant Metastasis
0	TIS	N0	M0
I	T1	N0	M0
	T2	N0	M0
II	T3	N0	M0
	T4	N0	M0
IIIA	T1,2	N1	M0
IIIB	T3,4	N1	M0
IIIC	Any T	N2	M0
IV	Any T	Any N	M1

4.2.7. Prognosis and treatment

CRC treatment options depend on a number of factors and mainly on the size, location and extent of the tumor, as well as the general condition of the patient. In the case of prognostic factors and unfavourable general health status, personalised treatments shall be identified. Useful tools for the treatment choice are the pre-operatory measurement of CEA (useful also for the follow-up), and of Ca 19.9 (largely used also if not supported by

strong scientific evidences) (Bast et al., 2001). As mentioned before, also an essential tool for the treatment is the TNM and staging determination. Although the determination of "T" and "N" factors in neoformations rarely changes the therapeutic approach, the determination of "M" component is essential in pre-operatory phase, as highlighted in the previous section, by CT, MRI and PET (Schmoll et al., 2012). Surgery is the most effective treatment; it consists in the removal of the mass, part of the healthy tissue surrounding it and the regional lymph nodes, the portion of the mass to remove is dependent to the tumor dimension, in some cases it is necessary a stoma. This is usually preceded by mechanical intestinal preparation, accompanied by a diet poor in slag and low in calories (Grossi, 2019). The type of surgery is carried out according to the location, the type and state of progress of the tumor, depending on this, the main procedures are: right hemicolectomy, transverse colon resection, left hemicolectomy, segmental colic resection and total colectomy (Grossi, 2019). The chemotherapy is often associated to the surgery, and it depends to the stage of the disease. Chemotherapy is always used in stage III and IV, while it is not used in stage I and is rarely used in stage II, for example in case of T4 (Cunningham et al., 2010). The most common used chemotherapeutics are fluorouracil, capecitabine, irinotecan and oxaliplatin. Fluoracil is an antimetabolite, analogous to thymine, thus acting by interfering with DNA synthesis. It is widely used in the treatment of cancers of the colon and pancreas and is administered paraenterally, usually combined with folic acid (McQuade et al., 2017). Capecitabine is taken orally, into the body is converted to fluoracil, that represent is active form (McQuade et al., 2017). Irinotecan, which is used in CRC and small cell lung cancer, belongs to the topoisomerase inhibitor family drugs. The binding between irinotecan and topoisomerase I induces DNA damage with consequent cell death (Fujita et al., 2015). Oxaliplatin, is able to interfere with the cell cycle at all levels, binding to DNA through the formation of cross-links between complementary filaments, often used in combination with fluoracil

(McQuade et al., 2017). It produces no significant increase in overall survival, but did produce an improvement in progression-free survival (de Gramont et al., 2000). This kind of treatment may represent the only treatment, when resection is not possible. In first-line therapy, antiangiogenic drugs such as bevacizumab are often added. Worthy of mention is another class of drugs used are EGFR inhibitors, of which the two approved, such as cetuximab and panitumumab (Shaib et al., 2013).

Radiotherapy (RT) consists in administration of ionizing radiation (IR) that induces the formation of intermediates ions and free radicals that causes DNA damages, with consequent cell death. This is one of the principal treatments against cancer, with around half of all patients receiving radiation therapy at some point during their management (Delaney et al., 2005). It is not a routinely therapy for the treatment of CRC, due to the sensitivity of the bowels to the radiations (DeVita et al., 2018). Radiotherapy can be used as neoadjuvant treatment in stage III CRC, helping to reduce the tumour size before to the surgery (Feeney et al., 2019), and for focally advanced rectal cancer this treatment is become a standard (Y. Li et al., 2016).

4.3. CANCER STEM CELLS

4.3.1. Definition and origin

The worst evolution of cancer is linked with the acquisition of malignant characteristics, which confer to the tumor the ability to spread in secondary sites, resist to therapies (chemo and radiotherapy) and to give recurrences. In the past decades, the classical model proposed for cancer was the stochastic model, according which each cell have the ability to initiate and promote the tumour evolution. Otherwise, nowadays the cancer stem cells (CSCs) model is more accepted, in particular in CRC. The CSCs theory shows its evidences starting from 1994 (Lapidot et al., 1994) but it was purposed for the first time most than 150 years ago (Cohnheim, 1867; Wicha et al., 2006) (Figure 12). According to

this model, the existence of a source cell in the tumour with stemness properties, able to proliferate and indefinitely maintain the growth due to its self-renewing ability. From this population, named CSC subpopulation, true architect of the tumour initiation and propagation, the tumour starts and propagates by clonal division (Fábián et al., 2009; Vaiopoulos et al., 2012). Furthermore, interacting with the micro environment, it is able to influence the cancer niche, and is in turn influenced by it, manifesting its own plasticity and the plasticity of the surrounding cells, which in turn can acquire stem character, and the CSCs like phenotype. This phenomena is named "CSCs dynamic model" (Hernández-Camarero et al., 2018; Markowitz & Bertagnolli, 2009a; Vermeulen et al., 2012; Zeuner et al., 2014; Y. Zhou et al., 2018).

Solid tumor masses enjoy of a high heterogeneity. The heterogeneity is present both between tumor cells and cells belonging to the stroma, useful for providing support, protection and nourishment to the organ or tumor mass. CSCs constitute a small fraction of the tumor component, but they are those that guarantee its maintenance, given their high ability to carry out self-renewal, and being tumorigenic. This characteristic is instead limited in the other tumoral cells that constitute the mass (Al-Hajj & Clarke, 2004; Batlle & Clevers, 2017; Toh et al., 2017).

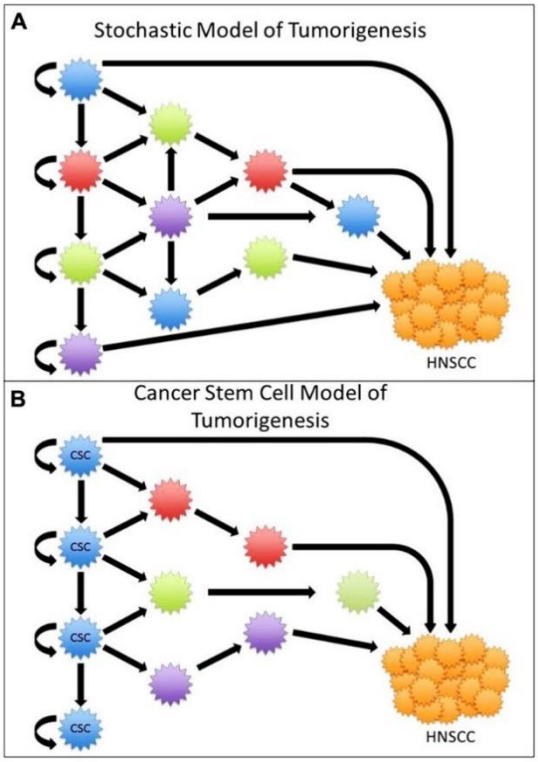


Figure 12. (A) In the stochastic model of tumorigenesis all cells are capable of renewal and tumorigenesis. (B) In the cancer stem cell (CSC) model, only a select group of cells are capable of asymmetric division and tumorigenesis. Source: (Chinn et al., 2012)

4.3.2. CSCs characteristics

CSCs are the tough guy of the tumour: they are characterized by self-renewal, multipotency, limitless proliferation potential, angiogenic, and immune evasion features. These mechanisms provide to metastasis, tumour recurrence and presents drug resistance and self-renewal. All these characteristics make them fundamental for the development of the tumor (Reya et al., 2001)

4.3.2.1. Epithelial to mesenchymal transition (EMT) and plasticity

EMT confers to the cells the ability to convert their phenotype from the epithelial to the mesenchymal one. This process is reversible and is regulated by stimuli from the microenvironment determined by the tumor niche, with the involvement of such factors such as, HGF, TGF-β, Wnt and IL6. During EMT, cells lose all the cell adhesions molecules, such as E-chaderin, with consequent losing of polarization. On the other hand, the cell assumes a simil-fibroblastic phenotype, becomes less sensitive to apoptosis,

expresses metalloproteases, fibronectin and typical mesenchymal markers, such as receptor for PDGF (platelet-derived grow factor), vimentin and N-chaderin (Figure 13). All these characteristics allow the cells to migrate from the original site, having a role in the spread of the tumor during the metastatic process (Batlle & Clevers, 2017; Mani et al., 2008; Thiery et al., 2009).

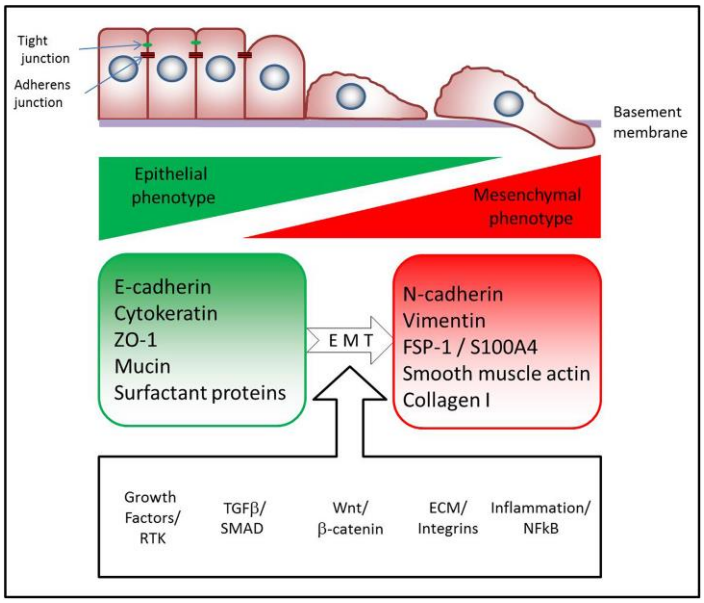


Figure 13. Epithelial to mesenchimal transition, with the factors involved in the process. Source(Bartis et al., 2014).

The EMT is linked with the plasticity phenomenon, which indicates the ability of the cells to convert from a phenotype to another. In tumor, plasticity is used from the cells to pass from a differentiated state to an undifferentiated one. This characteristic is particularly accentuated in CSCs, and have a critical role in the passage, from the origin of the CSCs to their metastatic potential (Hernández-Camarero et al., 2018; Marjanovic et al., 2013).

4.3.3. Quiescence

Quiescence is a cellular condition in which cells rest to G_0 phase of the cell cycle. This peculiarity of CSCs, allows them to avoid antitumor mechanisms implemented by the body and treatments. Furthermore, thanks to this mechanism the cells acquire the ability to trigger recurrence of the tumor (Batlle & Clevers, 2017; Reya et al., 2001).

4.3.4. Self-renewal and pluripotency

Self-renewal and pluripotency are two properties owned by stem cells and, therefore, also by CSCs (Hadjimichael et al., 2015). Self-renewal allows to the cells to maintain the same differentiating state for an indefinite number of divisions, this feature has an advantage for the CSC phenotype, ensuring a long living representation of it into the tumor. While the pluripotency confers to the cells the ability to differentiate in to any of the three germinal lines, ensuring the plasticity above mentioned (Squire, 2010).

These characteristics offer an advantage to the cells, and in the case of cancer cells, they increase the malignant potential. The pathways that play a role in these phenomena are Wnt, Notch and Hedgehog (Long et al., 2015). Other factors such as FGF in CRC (Otte et al., 2019), and some stem cell factors such as Sox2, cMyc, Oct3/4 and Klf4 (Merlos-Suárez et al., 2011; Takahashi & Yamanaka, 2006) are responsible of these stemness properties.

4.3.5. Therapeutic resistance

One of the most important issues in cancer is the therapeutic resistance to the treatment. In particular, by the above mentioned mechanisms, CSCs show a tenacious behaviour in respect to the other cells of the tumor and, in some cases when therapeutic treatment are applied, the tumor population results enriched in CSCs. There are three fundamental mechanisms by which CSCs resist to the chemotropic and radiotherapeutics treatments, upregulation of drug-efflux pumps, a high DNA-repair capacity, that decrease apoptosis and the interaction between CSC and microenvironment, or autophagy. DNA-damage repair genes are activated in CSCs in case to induced stress, such as DNA damage response including *NEK1*, *BRCA1*, *CHEK1*, *HUS1*, *UNG*, *XRCC5*, *SFPQ*, and *UHRF1*. Furthermore, these cells shows a greater checkpoint activation, demonstrated by the

activation of the ataxia-telangiectasia-mutated (AMT), Rad17, Chk1 and ChK2 proteins (Cojoc et al., 2015; Steinbichler et al., 2018). CSCs show an overexpression of ABC pump system, by which eliminates dangerous substances for the cells. Furthermore, this ability is used to conduce functional assay for CSC identification, as in the case of Hoechst exclusion assay (Kondo et al., 2004). Fourthly, autophagy is a mechanism that allows to the cells to provide to its own homeostasis also in presence of nutritional stress conditions. In this process the cell destroys its component in order to produces the necessary components. This process is activated by the release of factors that inhibit the path of PI3K, resulting in induction of autophagy and dormancy. This is a mechanism of survival during periods of cellular dormancy, puts in place to escape exogenous stress (Jo et al., 2008) (Steinbichler et al., 2018).

4.3.6. CSCs and CRC

There are many works that highlight the role of CSCs in the CRC. CSCs represent a small population, about 1% of the total tumour mass, located in the bottom of the intestinal crypts, region in which colorectal adenocarcinoma starts. Under normal conditions, the stem cells of the colon reside in the bottom of the colon crypts. One of the ways for the CSC formation is the degeneration of the stem cells as a result of genetic and epigenetic alterations, due to genetic mutations or microenvironment changing. Intestinal crypts structure and organization goes under the control of several pathways, which govern their differentiating and proliferative state, among others we find Hedgehog, Notch and Wnt. Wnt, in particular, is very important and is involved in maintaining the stemness states of the cells in the crypts. Wnt shows a gradual decrease moving from the bottom of the crypts to the surface, contrary to BMP (Medema & Vermeulen, 2011). As previously noted, alterations to the Wnt pathway, both internal to the cell, such as mutations of *APC*, or by hyperactivation by microenvironment, such as the hyper-expression of the HGF factor,

which has a promoter effect of this pathway, are at the base of the CRC onset. All this information taken together, can help us understand how alterations to the main regulatory pathways of intestinal homeostasis, may be the basis of the onset of CSCs, thus strengthening the evidence against their fundamental role in the birth, propagation and evolution of the CRC (Vries et al., 2010)(Haegebarth & Clevers, 2009). Other phenomena involved in this process can be seen, for example, in the loss of SMAD4 (Takaku et al., 1998), or in the hyperexpression of PI3K (Davies et al., 2011), just to name a few.

Colon CSCs have been widely characterized; in particular their identification can be performed by citofluorimetry, by analysing markers such as Epcam, CD44 and ALDH1 activity. Other analysable factors are positivity for LGR5, ABCG2, STAT3 and the overexpression of the stemness factors such as Sox2, Oct4, Klf4, c-Myc and Nanog (Munro et al., 2018).

4.4. MicroRNAs

miRNAs represent a family of short (18-25 nt) single strand (ss) endogenous RNA, whose fundamental role is to negatively regulate gene expression at post-transcriptional level in animals and plants. In particular, miRNAs act binding complementary sequences of mRNA and this bind induces their degradation or the inhibition of the translation. Therefore, miRNAs have a regulatory function of cell expression, having a role in both physiological and pathological conditions. In fact, they intervene in proliferation, apoptosis and cell differentiation, otherwise altered miRNA expression levels have been shown to drive a plethora of diseases, including cardiovascular diseases (Gupta et al., 2010), diabetes (Chakraborty et al., 2014), and cancer (Farace et al., 2020), in which they can act both as oncogenes (Gupta et al., 2010) and oncosuppressors (Cai et al., 2018). The genes coding the miRNAs are distributed on all the chromosomes, with the only exception for chromosome Y. In 50% of the cases the genes coding for different miRNAs are

localized in adjacent loci organized in clusters. At the genomic level, miRNA sequences can have different localizations. Intergenic miRNAs are found in genomic regions not occupied by known gene sequences, have their own promoter and can be monocistronic or polycistronic. Otherwise miRNAs can have intragenic localization, and it is supposed to be transcribed from the same promoter as their host genes and processed from the introns of host gene transcripts. They can be located both in protein coding and noncoding regions. When the pre-miRNA sequence occupies the whole intronic sequence, it is named miRtrons (Okamura et al., 2007). In some rare cases, miRNA sequence is located in exonic regions, and often overlap an exon and an intron of a noncoding gene, it is thought that these miRNAs use the promoter of the host gene (Rodriguez et al., 2004).

4.4.1. miRNA Biogenesis

The first product of the transcription of miRNA sequences are the primary miRNA (pri-miRNA), single-stranded sequences, several hundred bases long, which fold on themselves for complementarity, giving rise to a stem-lop structure (Figure 14). Pri-miRNAs have different origins, some are introns derived from the splicing of a mRNA, with which they share the promoter; others are extragenic and have their own promoters. In many cases, a single pri-miRNA contains a cluster of several microRNAs (Bartel, 2004). Into the nucleus, pri-miR after numerous cleavages, by the complex Drosha/Pasha, are maturated in pre-miRNA, lenght around 70 nucleotides. Pre-miRs are exported in the cytoplasm by Exportin 5 a GTP dependent protein (V. N. Kim, 2005). Once into the nucleus, pre-miR are processed by Dicer (RNasi III) to sequences with a lenght around 22 nt; it represent the mature miRNA (Figure 14). The complex represented by Dicer and the mature miRNA interacts with Ago1-Ago4 proteins, constituting miRISC (microRNA Induced Silencing Complex), which drives the miRNA to the complementary mRNA. Ago proteins belong to the Argonaute protein family, which bind RNA target with PAZ

and MID domains. RISC bind the RNA in the 3' UTR region, thanks to the complementarity of the miRNA (also if recent studies demonstrate the possibility to a bind of the miRNA also in codifying regions) (Bartel, 2004) (Figure 14). miRNAs act by different mechanisms, such as inducing of the RNA cleavage, while repression of RNA translation occurs in case of a minor complementarity between the miRNA and the mRNA sequence (Bartel, 2004). To induce RNA cleavage, it is necessary a high grade of complementarity between the miRNA and the mRNA sequence. In this case, the miRISC recruits endonucleases on the RNA target, and the mechanism starts in the cytoplasm and proceeds in specialized organelles named P-bodies, which represent the collection and disposal sites for transcripts. In the case of translate inhibition, the AGO subunits, interact competes with eIF4E for the 5' cap, avoiding the translation. In the eukaryotes, in fact, at the beginning of the translation, the cap in 5' is recognized by the proteins eIF4E, eIF4F, eIF4G; the latter, binding to the mRNA polyA tail-bound protein PABP1 favours the approach of the two ends of the transcript thus favouring the placement of the ribosome subunit 40S on the mRNA and initiating (Bartel, 2004; Filipowicz et al., 2008).

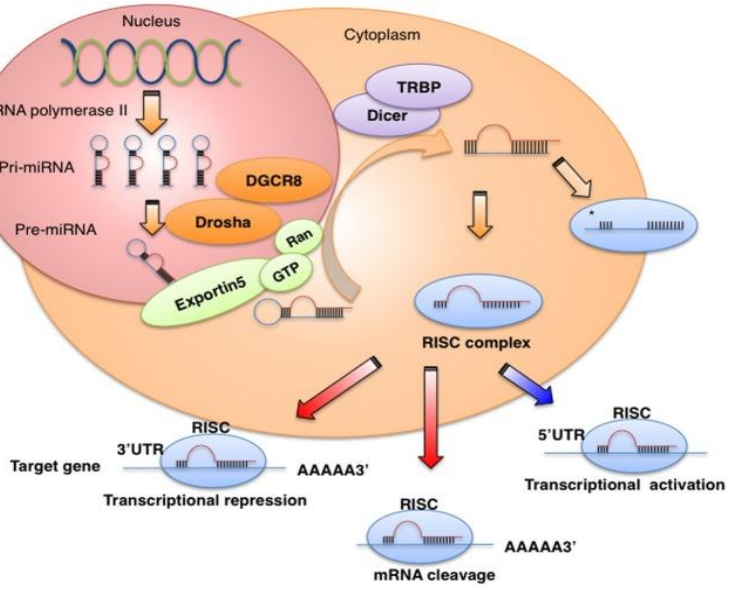


Figure 14. miRNAs' biosintesis

4.4.2. miRNA, cancer and CRC

miRNAs have a crucial role in cancer onset, progression and metastasis, as they can act both as oncomiR and as oncosuppressor miRNAs; furthermore, due to their high stability, they are investigated as a new promising biomarker class (Mitchell et al., 2008). An altered expression of the miRNAs is also reflected on the microenvironment, by which they are able to influence other cells. It has been demonstrated the role of miRNAs in the preparation of the secondary pre metastatic niche, that they reach by exosomes (Bebelman et al., 2018; Squadrito et al., 2014). The first evidence of miRNAs involvement in CRC was demonstrated in 2003 by Michael et al., who observed the reduction of expression of miR-143 and miR-145 in CRC in respect to normal colon tissue (Michael et al., 2003). This work is the first of a large literature about miRNAs alteration in CRC. Alterations in miRNAs expression have also been demonstrated in the Wnt pathway, the main CRC pathway, which is altered in 90% of CRCs. In particular, expression of miR135a/b, miR-494, miR-19a has been observed in association with suppression of *APC*. Also, an aberrant activation of the Wnt pathway has been associated with the altered expression of miR-21, miR-155, miR-103a, miR-1827, miR-145 and miR-34a. Furthermore, it has been observed the overexpression of some miRNAs that target other members of the Wnt pathways. Some examples are miR-224 that inhibit of *GSK3β* and promote the Wnt pathway (T. Li et al., 2016); miR-146a, that targets NUMB, guarantying cancer progression and metastasis (Hwang & Yang, 2016); miR-21 that targets *CTNNB1, TGFBR2, PI3KCA*, *BRAF*, *ZFHX3* and *SFRP1*, with a crucial role in tumor development and proliferation (Falzone et al., 2018; Fu et al., 2012). Otherwise some miRNAs turn out to be protective against CRC, and their downregulation is associated with CRC insurgence, such as in the case of miR43 and miR-145 accompanied by upregulation of *CD44, KLF4, KRAS* and *BRAF* (Pagliuca et al., 2013); miR-638 accompanied by the upregulation of *SOX2* and *TSPAN1*, with consequent augmented EMT, invasion,

migration and proliferative potential (K. Ma et al., 2014)(J. Zhang et al., 2014). Other miRNAs linked with the CRC progression that target an important tumor suppressor such as *TP53*, are miR-125b, miR-504, miR-29b, miR-30, miR-518 (Balacescu et al., 2018). On the other hand, in the 50% of CRC, overexpression of *EGFR* has been observed, and some studies suggest this overexpression in combination with the downregulation of some miRNAs considered oncosuppressor; it is the case of the above cited miR-143 and miR-145 (Pagliuca et al., 2013). Another important pathway in CRC is AKT-PI3K-mTOR, amplified in case of miR-126, miR-491 and miR-1 loss, or by upregulation of miR-21, miR-19 and miR-96 (Balacescu et al., 2018). As seen above, an important phenomenon in CRC prognosis is the activation of the EMT program (fundamental in CSC), some miRNAs has been associated with it, such as the overexpression of the oncomiR miR-675-5p in hypoxic conditions (Costa et al., 2017), or the downregulation of the oncoruppressors miR-34a, miR-375 and miR-192 (Balacescu et al., 2018). At last, some CRC miRNAs have a role in the insurgence of hepatocellular carcinoma. Among these we can find miR-21, miR-18a, miR328, miR17-5 and miR-92 (X. Luo et al., 2015; Mao et al., 2015; Sanchez-Mejias et al., 2019; Liang Wang et al., 2017; Yang et al., 2010; Y. Zhang et al., 2018).

5. HYPOTHESIS

The CRC is a disease characterized by a slow evolution, which affects a large part of the population, and an early diagnosis of it is the key factor for good prognosis. The importance of the CSCs in the prognosis of the CRC has been largely demonstrated by the literature. An important class of molecules involved in the regulation of cellular functions are the miRNAs. In fact, miRNAs deregulation can interfere with cellular homeostasis and lead to the development of various diseases, including cancer, and may have a role in the CSC phenotype.

The hypothesis of this work is that the identification of specific miRNAs, and the study of their alterations, can lead to the identification of specific molecular markers with a role in CRC, also through the regulation of the CSC phenotype. In case of hypothesis confirmation, these markers could help not only in the diagnostic and prognostic path, helping to find more precise and less invasive methods, but could guide towards a greater understanding of the CSC phenotype by providing practical help in combating CRC at clinical level by early screening of these potential biomarkers.

6. OBJECTIVES

The main objective of the present work is aimed to identify specific miRNAs with a role in CSCs of CRC, with the final scope to better understand the involvement of CSCs in CRC and to implement the diagnostic and prognostic methods.

To this purpose, we used two different approaches:

1. We select miRNAs from literature, known to have a role in CRC and/or in CSC phenotype. Then, we tried to understand their behaviour in vivo, studying these in patient's samples, and in vitro by cell models of healthy tissues, tumor tissues and CSCs.

2. In parallel, with a different approach, we tried to identify miRNAs with a role in CSC phenotype in CRC, independently from literature. To this hand, we started by studying the miRNoma in serum and stool of controls and CRC patients population. Then, we conducted a case-control meta-analysis for one candidate miRNA, followed by functional *in vitro* studies in cell models of CRC tumor and CSC.

7. MATERIAL AND METHODS

7.1. Cell culture

7.1.1. Cell line

The human colon fibroblast cell line CCD-18Co and the human CRC cell lines HCT-116 (with mutations in *CTNNB1*, *KRAS*, *PIK3CA*, *TGFR2*, *CDKN2A* and *BRCA2*), HT-29 (with mutations in *APC*, *BRAF*, *PIK3CA*, *SMAD4* and *TP53*) and T-84 (with mutations in *KRAS* and *TP53*) were purchased from American Type Culture Collection (ATCC, 10801, University Boulevard Manassas, VA 20110USA).

7.1.2. Culture conditions

Cell cultures were performed under sterile conditions, in a laminar flow hood, and were cultured under standard conditions in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St Louis, MO, USA) containing 10% of foetal bovine serum (FBS) (BioWhittaker, Lonza, Basel, Switzerland) and 1% penicillin/streptomycin (10,000 U / ml penicillin G and 10 mg / ml streptomycin, Sigma-Aldrich, St Louis, MO, USA) at 37° C under 5% CO2 and 90% humidity (Steri-Cult CO2 Incubator, Thermo Electron Corporation, Waltham, MA, USA). Every 48-72 hours the culture medium was changed. Cell culture with a maximum of 80-90% confluence was passed: the medium was collected and discarded, cells in adhesion were washed with phosphate buffered saline (PBS) (Lonza, Basel, Switzerland) and detached enzymatically using 1 mL trypsin-EDTA (0.25% (w/v) Trypsin + 0.53 mM EDTA; Sigma-Aldrich, St Louis, MO, USA), 5 minutes at incubation at 37°C. Then, trypsin was inactivated by the addition of complete culture media. Cells were spin down at 125 x g for 5 minutes (Centrifuge 5810R, Eppendorf Corp., Hamburg, Germany). Pellet cells were resuspended in pre-warmed culture media and reseeded into 75 or 25 cm^2 culture flasks. All cell lines were tested for authentication using the short-tandem repeat profiling and were passaged for less than 5 months, and routinely assayed for mycoplasma contamination.

7.1.2.1. CSCs enrichment

CSCs were obtained from HCT- 116, HT-29 and T-84 cell lines with the patented protocol WO2016020572A1 (Jiménez et al., 2018) which relies on the generation of colonospheres in Corning® Costar® Ultra-Low Attachment Six-Well Plates in DMEM/F-12 nutrient mixture without serum, supplemented with 1× B-27 (B-27™ Supplement [50×], Minus Vitamin A; Invitrogen), 4 ng/mL heparin (heparin sodium cell culture tested, Sigma), 10 μg/mL insulin (Insulin-Transferrin-Selenium [ITS-G] [100×], Invitrogen), 1 μg/mL hydrocortisone (Sigma), 10 ng/mL epidermal growth factor (Sigma), 10 ng/mL fibroblast growth factor (Sigma), 10 ng/mL interleukin 6 (Miltenyi) and 10 ng/mL hepatocellular growth factor (Miltenyi). After 72 h, the colonospheres were disaggregated by trypsinization as described before (section 4.1.2). The trypsin was inactivated by the addition of complete medium containing FBS 10%. The colonospheres were washed with PBS to remove traces of FBS, and the single cells were resuspended in new low-attachment plates with serum-free medium to generate secondary colonospheres.

7.1.3. CSCs characterization

7.1.3.1. Flow cytometry assay

ALDEFLUOR™ kit assay-fluorescein isothiocyanate (FITC) (Stem Cell Technologies, Vancouver, Canada) was used to detect ALDH1 activity. Diethylaminobenzaldehyde was used as an ALDH1 inhibitor to set the ALDH1 gates. The cell-surface levels of CD44 and CD326 were determined with anti-human CD44–phycoerythrin and CD326–fluorescein isothiocyanate antibodies (Miltenyi Biotec). All samples were analysed with a FACSCanto™ II flow cytometer (BD Biosciences) using the FACSDiva™ software. The brightly fluorescent PE, APC and FITC were detected in the red (564-606 nm), blue (650-670 nm) and green (520-540 nm) fluorescence channels respectively. In brief, cells were washed with PBS and resuspended in 100 μL of blocking buffer, composed as follow: 1%

of bovine serum albumin (BSA) (Sigma-Aldrich, St Louis, MO, USA) and 2mM EDTA in PBS; in case of ALDH1, cells were resuspended in ALDEFLUOR assay buffer and cells were spinned down and 6 μL of anti-human antibodies or 5 μL of ALDH1 were added to the cell suspension for CRC cell lines in the different conditions. Incubation with CD44 and CD326 antibodies was performed for 10 minutes at 4°C in the dark; incubation with ALDH1 was 30 minutes at 37°C in the dark. Then, cells were centrifuged at 4°C and resuspended in cold PBS.

7.1.3.2. Hoechst assay

The Analysis with Hoechst 33342 (Sigma Aldrich; St Louis, MO, USA) dye, allowed to identify the cells overexpressing ABC transporters with the capacity to efflux drugs. Hoechst was added to 1 x 106 cells/mL, resuspended in DMEM with 10% FBS, to a final concentration of 5 μg/ml, and incubated in the dark at 37°C for 90 minutes. 5μM verapamil (Sigma-Aldrich) were used for negative controls, to maintain the efflux channel closed inhibiting the capacity to efflux Hoechst 33342 by cells. After these 90 min cells were spin down at 4°C and resuspended in cold PBS. The brightly fluorescent Hoechst 33342-positive cells were measured by flow cytometry in Hoesch blue (440/40) and Hoesch red (695/40) channels of a FACScan Aria III and data were analyzed using FACS DIVA.

7.1.4. Cell cryopreservation and recovery

Cell lines were stored *via* freezing. The culture medium was discarded and cells were washed twice with PBS and trypsinized as showed before (section 6.1.2.1). Then, trypsin-EDTA was inactivated by adding complete medium and the cell suspension was collected into sterile 30 mL tubes. Cells were centrifuged 5 min at 125 x g, and suspended in freezing medium containing 93% FBS and 7% Dimethyl Sulfoxide (DMSO) (Sigma- Aldrich, St

Louis, MO, USA) and 1 mL of total volume was transferred into 1.5 mL cryotubes (Nunc, Thermo Fisher Scientific, Waltham, MA, USA). Cryovials containing the cells were placed into an isopropanol cryo-freezing container (Sigma-Aldrich, St Louis, MO, USA) at -80ºC for short periods; and transferred at -180°C in case of long period's time.

In order to restore the cell lines stored at -80 ° C or -180°C, these thawed at 37 ºC in a water bath and suspended in fresh sterile culture medium, then centrifuged at 125 x g for 5 min to remove DMSO residues (two washes). Cells pellets were resuspended in culture media and seeded into a 25 cm^2 culture flask.

7.1.5. Cell count

For the cell count, an aliquot of cells was diluted 1:1 with 0.4% trypan bleu solution (Sigma-Aldrich, St Louis, MO, USA), then 10 µL were loaded in the Neubauer chamber. To obtain the number of cells per millilitre, live cells, not coloured by the trypan bleu, were counted in 4 squares, the number obtained was divided by 4, and multiplied by 10,000 and finally, then divided by 2, the dilution factor. The concentration was multiplied for the number of mL, in order to know the cell amount.

7.2. Gene expression in cell line

7.2.1. RNA isolation from cell line

Cells were disaggregated with trypsin, pelleted at 1500 × g for 5 min, and washed twice in cold PBS. One ml of TRI Reagent® (Sigma-Aldrich) was added to the cells pellets and the cells were moved to 1 ml Eppendorf microtubes and allowed to stand at room temperature (RT) for 15 min. Then, 200 µl chloroform was added and the cells were vortexed for 15 s and incubated at RT for 10 min. The cells were centrifuged at 12,000 × g for 10 min at 4° C, the upper aqueous phase of the solution was transferred into a new Eppendorf microtube. Isopropanol (500 µl) was added to the aqueous phase, which was then vortexed, incubated at RT for 10 min, and centrifuged at 12,000 × g for 10 min at

4°C. The isopropanol was removed and replaced with 1 ml of ethanol (EtOH, 75% in Milli-Q™ water) before centrifugation at 17,000 × g for 5 min at 4° C. After the EtOH was removed, the sample was left at RT for 1 h and then resuspended in 50 µl of Milli-Q™ water. The RNA concentrations and quality were evaluated routinely with a NanoDrop spectrophotometer (Thermo Fisher Scientific).

7.2.2. RNA quantification

For quantification of RNA, we proceeded to the reading of the absorbance at 260 and 280 nm using a NanoDrop (NanoDrop™ 2000/2000c Spectrophotometers, Thermo Scientific ™). The OD260/OD280 relationship allowed us to calculate the purity of nucleic acids, whereas an optimal range of values between 1.8 and 2. RNA concentration was calculated considering an OD unit at 260 nm corresponds to a concentration of 40 mg/ml nucleic acid. The purified RNA samples were placed at 80°C for long-term storage.

7.2.3. Retro transcription

The RNA samples were diluted in nuclease-free water and resuspended to a final concentration of 5 ng/µl. cDNA was synthesized with the miRCURY™ LNA™ RT Kit (Qiagen), according to the manufacturer's instructions. The reactions were spiked with exogenous UniSp6 RNA (RNA Spike-In Kit, Qiagen). The following RT protocol was applied: 60 min at 42° C, 5 min at 95° C; immediate cooling to 4° C; storage at -20° C until processing.

7.2.4. Real Time RT-PCR for miRNA in cell lines

The RNA from HCT-116, HT-29, and T-84 cell monolayers and their respective colonospheres were diluted in nuclease-free water and resuspended at 5 ng/µL final concentration. cDNA was synthesised with the miRCURY™ LNA™ RT Kit (Qiagen), according to the manufacturer's instructions. The reactions were spiked with exogenous UniSp6 RNA (RNA Spike-In Kit, Qiagen). miRCURY LNA miRNA primers (Qiagen)

were used for both the approaches. For the analyses on the miRNAs selected from literature, commercial pair primers for hsa-miR-221-3p, hsa-miR-34a-5p, hsa-miR-10b-5p, hsa-miR-18a-5p, hsa-miR-210-3p, hsa-miR-31-5p, hsa-miR-16-3p, and hsa-miR-21-5p were used. While for the analyses of candidate miRNA derived from the study of the miRNome, commercial pair primers for hsa-miR-486-5p was used. For normalization in both the approaches, the U6 housekeeping gene was used. For the real-time RT-PCR assays, the cDNA was diluted 1:80 in nuclease-free water and 4 µL of diluted cDNA was mixed with 5 µL of PCR master mix and 1 µL of each primer pair. The thermal cycling conditions included a melting curve analysis and were run in the StepOne Real-Time PCR System (Thermo Fisher, Waltham, MA, Italy) with the following parameters: 95 °C for 10 min, followed by 45 cycles at 95 °C for 10 s and 60 °C for 1 min, with a ramp rate of 1.6 °C/s.

7.2.5. Transient transfection with synthetic miR-486-5p mimics and inhibitors

To increase or decrease levels of miR-486-5p into the HCT-116 cell line, miR-486-5p mimic or inhibitor (Qiagen) was used, paired with the relative controls. The 50-FAM-fluorescence-labelled delivery control by Qiagen was used to measure the transfection efficiency in the cell models. Transfection was performed with TransIT-X2® Transfection Reagent (Mirus Bio, Madison, WI, USA), according to the manufacturer's instructions. The miRNA mimic and inhibitor (final concentrations of 5 nM and 50 nM, respectively) were prepared in 1.5 µL of TransIT-X2® and were transfected using 100 µL of Opti-MEM medium (Gibco, New York, NY, USA) into each well of standard 24-well plates containing 6 x 10^3 cells in 0.4 mL of medium. Before further analysis, the cells were cultured for 3 days at 37° C under 5% CO2.

7.2.6. Analysis for stemnes gene expression

Total RNA was extracted from HCT-116 monolayer cells and CSCs as described in

Section 6.2.1., before and after treatment with the miR-486-5p mimic or inhibitor, to evaluate the effect of miR-486-5p on the expression levels of stemness factors (SOX2, NANOG, KLF4, OCT4, and cMYC). The cDNA (diluted 1:80), GoTaq® qPCR Master Mix (Promega), and primers from the StemElite. (Promega) SOX2/NANOG/KLF4/OCT4/cMYC/GAPDH Primer Pair (20X) were used according to the manufacturer's instructions. GAPDH was used as the housekeeping gene. For details about the protocol see section 6.2.4.

7.2.7. Microarray hybridization

The total RNA extracted from the HCT-116 monolayer and CSCs after treatment with the miR-486-5p mimic or inhibitor was amplified, labelled, and hybridised with the Clariom D platform (Affymetrix, Santa Clara, CA, USA), according to the manufacturer's instructions. Briefly, a HeLa positive control and polyA RNA controls were prepared according to the manufacturer's instructions. The Affymetrix GeneChip WT Pico Kit was used to synthesise single-stranded cDNA with the T7 promoter sequence at the 5' and using 3 ng of total RNA for each condition (mimic, mimic control, inhibitor, and inhibitor control, in triplicate). A 3' adaptor was added to the single-stranded cDNA, and double-stranded cDNA was synthesised with Taq DNA polymerase and adaptor-specific primers. cRNA was synthesised with T7 RNA polymerase and purified with purification beads. The cRNA was used to synthesise second-cycle single-stranded cDNA. The samples were then subjected to RNA hydrolysis with RNase H and the purification, fragmentation, and labelling of the second-cycle single-stranded cDNA. The Affymetrix GeneChip® Hybridization, Wash, and Stain Kit were used to process the array. The biotin-labelled single-stranded cDNA was hybridised to the GeneChip® Cartridge Array, and the array was incubated for 16 h in an Affymetrix GeneChip® Hybridization Oven 645 at 45 °C with rotation at 60 rpm. The array was scanned with the Affymetrix Expression Console.

software (v.1.4; Affymetrix London, UK). The working hypothesis that miR-486-5p affects one or more of the principal genes involved in one of the four principal pathways of the CSC phenotype (Wnt, Notch, Hedgehog, and TGF-β), with the same trends in the monolayer cells and CSCs, was then tested.

7.3. Patients

Informed consent for use of human samples was obtained by the participant to the study, and the study was approved by the ethic committee. The staging was evaluated by TNM classification, according to AIOM, guidelines (Grossi, 2019).

7.3.1. Patients sampling

As mentioned before two approaches were used in this study, one relative to miRNAs selected from literature, conduced on 12 patients, and one in which we started from information derived from miRNome analyses of the serum of 47 patients and further investigated in stool derived from 135 individuals. The population used to test the miRNAs choose by literature was composed by 12 patients, from which three different kinds of samples were taken: serum (S), healthy tissue (HT) and CRC tissue (CT). These patients were paired in function of presence or absence of metastases or in case of high (stage from IIIb to IV) or low (stage from 0 to IIIA) stage. Before surgery or any treatment, 5 ml of blood were collected from the patients and transferred to Serum-Gel Clotting Activator S-Monovette ™ (9 ml tube, Sarstedt), incubated at RT for 60 min, and centrifuged to obtain serum aliquots. During the surgical removal of the tumor mass, samples of colorectal cancer and healthy biopsy tissue were obtained from the CRC patients and immediately transferred in RNAlater-filled 15 ml tubes (Sigma). After 24 h at 4° C, the RNAlater was removed from the tissues' samples and the dried samples were stored at −80° C until processing.

For the second approach, independent from literature, two distinct population were used. One was composed by 47 individuals from which serum was obtained as mentioned above in this paragraph, this population was divided into three groups: controls (non-tumor n = 10; average age = 64.6 years; 60% male, 40% female), CRC (patients with stage I – II colorectal adenocarcinoma without metastasis, n = 13; average age = 70.6 years; 46.2% male, 53.8% female), and metastatic CRC (patients with stage III – IV colorectal adenocarcinoma with both lymphatic and distant metastasis, n = 24; average age = 69.6 years; 54.2% male, 45.8% female). In parallel in an independent population, we performed small RNA-sequencing to analyse the whole miRNome in 137 stools samples from 79 healthy subjects (mean age = 57.4 years; 50.7% male, 49.3% female) and 58 CRC patients (mean age = 71 years; 70.7% male, 29.3% female). Naturally, evacuated stool samples were obtained from all patients after they were instructed to self-collect the specimens at home before any bowel preparation for colonoscopy. Stool were collected in the Stool Nucleic Acid Collection and Preservation Tubes (Norgen Biotek Corp., Thorold, ON, Canada) with RNA stabilizing solution, and returned to the hospital the day of the colonoscopy or at the time of blood sampling. Aliquots (200 mL) of the stool samples were stored at 80° C until RNA and DNA extraction. The study was approved by the local Ethic Committee (Azienda Ospedaliera SS, Antonio e Biagio e C. Arrigo of Alessandria, Italy), and informed consent was obtained from all the participants.

7.4. RNA Extraction from Patient's tissues

The cancerous and healthy tissues (30 mg) were cut into thin pieces with a scalpel in Petri's dishes and transferred into 2 ml vials containing 700 µl of QIAzol Lysis Reagent (Qiagen) and one stainless steel bead (ø = 7 mm). The tissues were homogenized with a TissueLyser LT (Qiagen) at 40 oscillations/min for 2 min. The RNA was extracted by miRNeasy® Mini Kit (Qiagen), according to the manufacturer's instructions. In brief,

140µl of chloroform was added and the samples were subjected to a vigorous agitation for 15 seconds using a vortex. Further incubation of the tissues at room temperature was then carried out for 2-3 minutes. Then, the samples were centrifuged at 12000g, at 4º C, for 15 minutes. During the centrifugation, two phases were formed: the aqueous phase at the top and the organic phase at the bottom. The aqueous phase (about 350 µl) was taken and deposited in a new 2-ml eppendorf, to which 1.5 volumes of 100% Etoh (525 µl) were added and mixed. 700µl of the solution thus obtained were transferred into a mini-column RNeasy, inserted in a 2ml tube. The columns were then subjected to centrifugation at a speed > 8000g, at room temperature for 15 seconds. The flow-through was discarded and the centrifugation was repeated with the rest of the solution. 700µl of RWT buffers were added to the columns, which were centrifuged under the same conditions as above. The flow-through was eliminated and 500µl of buffer RPE was added. At this point, the columns were centrifuged at a speed of 8000g, at room temperature, for 2 minutes. The flow-trough was again eliminated and the columns were placed into a new 2 ml eppendorf and centrifuged at maximum speed for 1 minute. Subsequently, the columns were placed inside new 1.5 ml eppendorf and to these were added 30-50µl of RNase-free water. Finally, further centrifugation was repeated at a speed of 8000g, for 1 minute at room temperature. The RNA concentrations and quality were determined with a NanoDrop spectrophotometer as described before 6.2.2.

7.5. RNA Extraction from Patient's serum

The blood samples were centrifuged at 1900 × g for 10 min at 4 ° C. The serum in the upper phase was transferred to new tubes and centrifuged at 16,000 × g for 10 min at 4 ° C. The supernatant was transferred to a new 1.5 ml tubes and stored at -80° C until processing. After the samples were thawed at RT for 5 min, five volumes of QIAzol Lysis Reagent were added to each sample and incubated at RT for 5 min, before the miRNAs

were extracted by miRNeasy® Serum / Plasma Kit (Qiagen), according to the manufacturer's instructions. The blood samples collected in the Sarstedt S-Monovette® Serum-Gel were centrifuged at 1,900 × g for 10 min at 4 ° C, this operation allowed to separate the serum in the upper part of the vial. The serum in the upper phase was transferred to new tubes and centrifuged at 16,000 × g for 10 min at 4 ° C, to remove the cellular debris. The supernatant was transferred to a new 1.5 ml tubes and stored at -80 °C until processing. For the miRNA isolation the samples were placed under a chemical hood, and when thawed, 5 volumes of the QIAzol lysis reagent were added. The solution obtained was incubated for five minutes at room temperature to dissociate nucleoprotein complexes. Subsequently, 3.5 µl of Spike-in Control miRNA ($1.6x10^8$ copies/1 µl of work solution) were added and mixed; 1 volume of chloroform was added and the solution was shaken vigorously for 15 seconds (200µl of plasma - 200µl of chloroform). The solution was incubated for 3 minutes at room temperature. Subsequently, the samples were centrifuged at 12000g, 4°C, 15', to allow the formation of the two phases (the upper aqueous phase and the lower organic phase). Then the aqueous solution was taken and transferred to a new 2-ml eppendorf in which 1.5 volumes of Et-OH-100% were added; the whole was thoroughly mixed. The entire sample (maximum 770 µl) was transferred to the 2ml RNeasy mini column. The samples were again centrifuged at >8000g, 15 seconds at 25° C, and the flow-trough that settled on the bottom of the column was eliminated. They were added to the RWT buffer column 700µl; the columns were centrifuged at 8000g, 15 minutes, 25° C, and the flow-trough that settled on the bottom of the column was discarded again.

7.6. RNA Extraction from Patient's stool

The collected stools RNA were extracted from the stool samples with a Stool Total RNA Purification Kit (Norgen Biotek Corp.). In brief for the lysate preparation, 200 mg of stool

sample and 1 mL of Lysis Buffer C were moved into a Bead Tube, it was briefly vortexed to mix stool and Lysis Buffer. The tube was secured horizontally on a flat-bed vortex pad with tape and vortexed for 5 min at maximum speed. Then the tubes were centrifuged for 3 min at 14000 × g. Then 600 µL of supernatant were transferred to an RNAase-free microcentrifuge tube, and an equal volume of 70% ethanol was added to the lysate, and the solution was votexed. Up to 600 µL of the clarified lysate with ethanol were transferred to a spin column inserted into a collection tube and centrifuge for 1 minute at 14000 × g. The flowthrough was discarded and the spin column was reassembled with the collection tube. This passage was repeated twice in order to collect to the column the entire sample. Then 400 µL of Wash Solution A were added to the column and centrifuge for 1 minute. The flowthrough was discarded and the spin column was reassembled with its collection tube. These passages were repeated for three times. Then the spin column was dried by centrifuging it for 2 minutes, and the collection tube was discarded. In order to collect the eluate, the column was placed into a fresh 1.7 mL elution tube, and 75 µL of Elution Buffer E were added to the column. The column containing the 75 µL of Elution Buffer E was centrifuged for 2 minutes at 200 x g, followed by a 1 minute spin at 14,000 x g. The purified RNA was stored at –70°C until use.

7.7. Small RNA sequencing in serum

To analyze the expression levels of the whole miRNome in different tumor phases, we pooled the RNAs extracted from the sera from the three groups: non-tumor (n = 10), tumor (n = 13), and metastatic (n = 24). The final sample from each group had a final volume of 11 L and a yield of 1 g of RNA, and each initial sample contributed with an equal volume to the final pool. The pools were analyzed in duplicate in service at the Center for Genomics and Oncological Research (GENYO; Pfizer – University of Granada – Andalusian Regional Government, Granada, Spain). Standard quality control steps were

included to determine the quantity and quality of the total RNA with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and electrophoresis on an Agilent 2100 Bioanalyzer, with the Eukaryote Total RNA Pico Kit (Agilent Technologies, Santa Clara, CA, USA), and a High Sensitivity DNA Assay (Agilent Technologies). Libraries were prepared from 50 ng of extracted RNA with the NEXTflex— Small RNA-Seq Kit v2 (Bioo Scientific Corp., Austin, TX, USA). To prepare small-RNA libraries, we simultaneously multiplexed 12 samples using 22 cycles of amplification and the miRNAs were selected by size (130–170 bp; or 16–56 bp without adapters) in an acrylamide gel. All the libraries were sequenced with the NextSeq 550 System (Illumina, San Diego, CA, USA) using a High Output Kit v2 (Illumina) with 75 cycles. All samples were read in a single run.

7.8. Small RNA sequencing in stool

Library preparation from total RNA extracted from stool was performed with a NEBNext multiplex small RNA library prep set for Illumina. For each sample, 250 ng of RNA were used as the starting material to prepare libraries. Each library was prepared with a unique indexed primer so that the libraries could all be pooled into one sequencing lane. Multiplex adapter ligations, reverse transcription primer hybridization, reverse transcription reactions, and the PCR amplification was performed as described in the protocol provided by the manufacturer. After PCR preamplification, the cDNA constructs were purified with a QIAQuick PCR purification kit (Qiagen, Germany) following the modifications suggested in the NEBNext multiplex small RNA library prep protocol. Further quality control checks and size selections were performed following the NEBNext multiplex small RNA library prep protocol (protocol E7330; New England BioLabs Inc., USA). Size selection of the amplified cDNA constructs was performed using Novex Tris-borate-EDTA (TBE) gels (Invitrogen) (6%) and following the procedure of gel

electrophoresis running and purification of the construct described in the Illumina TruSeq small RNA library prep protocol. The 140-nt and 150-nt bands correspond to adapter-ligated constructs derived from RNA fragments of 21 to 30 nt. A concluding Bioanalyzer 2100 run performed with a high-sensitivity DNA kit (Agilent Technologies, Germany) permitted checking the final size, purity, and concentration for the sequences in the DNA libraries. The obtained libraries (24 samples multiplex) were subjected to the Illumina sequencing pipeline, passing through clonal cluster generation on a single-read flow cell (Illumina Inc., USA) by bridge amplification on a cBot (TruSeq SR cluster kit, v3-cBOT-HS; Illumina Inc., USA) and 50 cycles of sequencing by synthesis using a HiSeq 2000 sequencer (Illumina Inc., USA) (in collaboration with Genecore Facility at EMBL, Heidelberg, Germany).

7.9. Real-Time RT-PCR Assay for miRNA Expression Profiling

RNA samples from each patient were diluted in nuclease-free water to a final concentration of 5 ng/µL and cDNA was synthesized with the miRCURY-LNA-RT Kit (Qiagen), according to the manufacturer's instructions. The reactions were spiked in with exogenous UniSp6 RNA (RNA Spike-In Kit; Qiagen). The following RT protocol was used: 60 minutes at 42° C, 5 minutes at 95° C, and cooling to 4° C. The cDNA was immediately stored at 20° C until processing. To investigate in the three tissues healthy tissue, cancer tissus and serum the expression of literature derived miRNAs with a role in CRC, the Real-Time LNA™ PCR primer set (Qiagen) was used as follows: hsa-miR-221-3p, hsa-miR-34a-5p, hsa-miR-10b-5p, hsa-miR-18a-5p, hsa-miR-210-3p, hsa-miR-31-5p, hsa-miR-16-3p, and hsa-miR-21-5p, using for normalization miR-24-3p. While for the literature independent analysis in serum, miRCURY LNA miRNA Custom PCR Panels (Qiagen) furnished pre-spotted with the primers hsa-miR-223-3p, hsa-miR-93-5p, miR-486-5p, and hsa-miR-342-3p in 96-well plates were used; in this case, two kind of

normalization were done, one using as housekeeping gene the has-miR-342-3p (Kok et al., 2015), and one using the average of the cycle threshold (Ct) values of hsa-miR-223-3p, hsa-miR-93-5p, and hsa-miR-342-3p, according to the method described by Mestdagh et al. (Mestdagh et al., 2009). In the real-time RT-PCR assays, the cDNA was diluted 1:40 in nuclease-free water and 4µL of diluted cDNA were mixed with 5 µL of PCR Master Mix and 1 µL of each primer (when not pre-spotted in the plate). The thermal cycling conditions included a melting curve analysis and were run in the StepOne Real-Time PCR System (Thermo Fisher, Waltham, MA, Italy) with the following parameters: 95° C for 10 min, followed by 45 cycles at 95° C for 10 s and 60° C for 1 min, with a ramp rate of 1.6° C/s. For the normalization of the miRNA expression, were used two different approaches, one for the normalization of the known miRNAs and one for the normalization of ex-novo investigation.

7.10. Meta-Analysis of miR486-5p Serum and Tissues from public Database

Through the use of the database GEOdataset (Barrett et al., 2013), ten datasets relative to the expression levels of miRNAs in CRC patients and controls were identified. Five datasets were referred to the miRNA expression levels in serum and five were referred to the expression in colon tissue. The serum datasets were: gse59856 (151 healthy individuals and 50 CRC patients), gse106817 (2724 healthy individuals and 150 CRC patients), gse112264 (41 healthy individuals and 50 CRC patients), gse113486 (100 healthy individuals and 40 CRC patients), and gse124158 (275 healthy individuals and 30 CRC patients). The tissue datasets were: gse39845 (3 healthy colon tissues and 3 cancerous colon tissues), gse41655 (74 healthy colon tissues and 33 cancerous colon tissues), gse45349 (4 healthy colon tissues and 4 cancerous colon tissues), gse115513 (763 healthy colon tissues and 750 cancerous colon tissues), and gse136020 (6 healthy colon tissues and 8 cancerous colon tissues). Once identified the datasets, the "*minimal*

formatted family file" containing two different files, one relating to the detection of miRNAs per patient and one relating to the identification codes attributed to each individual miRNA, the information derived from the two files were merged in a unique file and analysed as described in statistical analyses.

Statistics

7.10.1. Statistics approach 1

In the in vitro assays, we compared the CRC cells (both CSCs and monolayers) with the normal CCD18Co cell line. Then the CSCs were compared with the corresponding monolayers. To determine the miRNAs most frequent associated with CSC phenotype, their expression was determinate in the three CSC models as the geometric means of the FC values. Analysis of the PCR dataset was performed with the software supplied with the 7500 Real Time PCR System (Applied Biosystems). The real-time RT–PCR assays were run in triplicate and the mean Ct value for each miRNA under each set of conditions was used to determine FC with the $2^{-\Delta\Delta Ct}$ method, using the Ct values for the hsa-miR-24 reference miRNA in data normalization. The FC values were compared with the Student's independent t-test. A two-sided p value of < 0.05 was considered statistically significant.

In each patient, we compared the miRNA levels in the serum and cancer tissue with those in the healthy tissue from the same patient. The average values for replicate spots of each miRNA were background subtracted and normalized. The patients were also stratified according to cancer grade and the presence/absence of metastasis, and appropriate statistical analyses were performed and data analysed with Microsoft Excel 10. The FCs were graphed as histograms on a logarithmic-scale Y axis (base 10). All statistical analyses were performed with Stata statistical software: release 15 (StataCorp LLC, College Station, TX, USA). Descriptive statistics were primarily used in the analysis of the data. The

normality of the distributions of the miRNAs was tested with the Shapiro–Wilk test, and for normally distributed data, Student's two-sided t-test was used where stated. The non-parametric Mann–Whitney U test followed by the two-sided Dunn's pairwise post hoc test based on rank sums, with the Benjamini–Hochberg adjustment of p values (p), was used to compare miRNA expression. Spearman's rho was used to assess the correlations between the miRNA expression levels and the clinicopathological parameters of the patients, including metastasis, tumour grading, GGT, ALT, AST, CPK, cholesterol, HDL, LDL, triglycerides, glycaemic status, age and sex.

7.10.2. Statistical analysis approach 2

To evaluate the whole serum miRNome differential expression in sera of CRC patients under different conditions and in the controls, the obtained sequences were mapped onto the reference human genome hg38 version (accession GCA_000001405.15) (Zheng-Bradley et al., 2017), and the raw data were normalised with the trimmed mean of M values (TMM) method (Robinson & Oshlack, 2010). The fold change (FC) values were compared with Student's independent t test and analysis of variance (ANOVA). A two-sided p value < 0.05 was considered statistically significant, and the false discovery rate (FDR) (Benjamini & Hochberg, 1995) was calculated to account for multiple comparisons. Real-time RT–PCR was used to evaluate the differential expression of miR-486-5p in the sera of the different groups of patients and controls, and its differential expression in the three cell models under the two sets of conditions examined. The same technique was also used to test the differential expression of stemness factors in HCT-116 cells (monolayers and CSCs) after treatment with the miR-486-5p mimic or inhibitor. The real-time RT–PCR assays were run in triplicate, and the mean cycle threshold (Ct) value for each miRNA under each set of conditions was used to determine the FC with the $2^{-\Delta\Delta Ct}$ method. The FCs were compared with Student's unpaired t test. A two-sided p value

< 0.05 was considered statistically significant. To extend the population of study, a random-effects meta-analysis was performed by computing the log FC of miR-486-5p in CRC patients vs. controls. To evaluate the miRNome in stool samples, small-RNA-Seq pipeline analyses were performed with a previously described approach (Tarallo et al., 2019). The differential expression analysis was performed with the DESeq2 R package v.1.22.2 (Love et al., 2014) using the likelihood ratio test (LRT) function, adjusting for age and sex as covariates. A gene was defined as differentially expressed if it was associated with a Benjamini–Hochberg (BH)-adjusted p value < 0.05 and was supported by at least a median number of reads > 20 within at least one of the sample groups considered. Data from microarray were normalised with the Affymetrix Expression Console™ software (v.1.4; Affymetrix UK Ltd.), which provides signal estimation and quality control functionalities for the GeneChip Expression Arrays, and the Affymetrix Transcriptome Analysis Console (TAC) Software version 4.0.2 (Thermo Fisher). We performed a genome-wide scan to identify those genes with expression patterns consistent with the working hypothesis derived from the genomic analysis, the meta-analysis, and from functional analysis of HCT-116. To this end, after the treatments with mimics or inhibitor of miR-486-5p, we identified genes that were progressively inhibited or promoted by miR-486-5p, both in monolayer and CSC, by using the Jonckheere–Terpstra test for trend, with the empirical p value determined after 10,000 permutations. Subsequently, we investigated whether the selected genes were enriched in the four main pathways of the CSCs phenotype (Wnt, Hedgehog, Notch and TGF-β), using the weighted Kolmogorov–Smirnov enrichment test (Charmpi & Ycart, 2015), considering enrichment p-value <0.05 as statically significant.

8. RESULTS

Results will be presented divided into two parts. “Results section 1”, includes the results about the first approach, regarding the study of a selection of miRNAs derived from the literature in three kind of samples from a small population of patients, and cell models. “Results section 2” includes the results about the second approach, in which starting from the study of the miRNome in serum of control and patients, we individuate one miRNA suitable as a diagnostic and prognostic marker and characterized its role in gene regulation of CSCs.

8.1. Results section 1

8.1.1. Cells

8.1.1.1. CSC enrichment in established human CRC cell lines HCT-116, HT-29 and T-84

In all the cell models, HCT-116, HT-29 and T-84 CRC, the cancer stem cell enrichment was achieved after secondary colonosphere formation. After 4 days of CSCs enrichment treatment, colonsphere with a diameter (ø)> 200 μm after 4 days were formed, these cells were disaggregated and passed to form secondary colonsphere at the 6^{th} day (Figure 15A). The aldehyde dehydrogenase (ALDH1) CSC marker was evaluated with flow cytometry in cells derived from colonospheres and compared to ALDH1 in the cell lines cultured in adherent condition (monolayers, Figure 15B). ALDH1 activity increased significantly from 54.2% in the HCT-116 monolayer to 91.3% in the HCT-116 colonospheres ($p < 0.05$); from 9.25% in the HT-29 monolayer to 83.2% in the HT-29 colonospheres ($p < 0.005$); and from 4.5% in the T-84 monolayer to 31% in the T-84 colonospheres ($p < 0.005$) (Figure 15C). The positivity to CD44 / CD326 was also determined (Figure 15D), and increased from 58% in the HCT-116 monolayer to 85.2% in the HCT-116 colonospheres; from 22.2% in the HT-29 monolayer to 100% in the HT-29 colonospheres ($p < 0.005$) and from 0.9% in the T-84 monolayer to 15.4% in the T-84 colonospheres ($p < 0.005$) (Figures 15E).

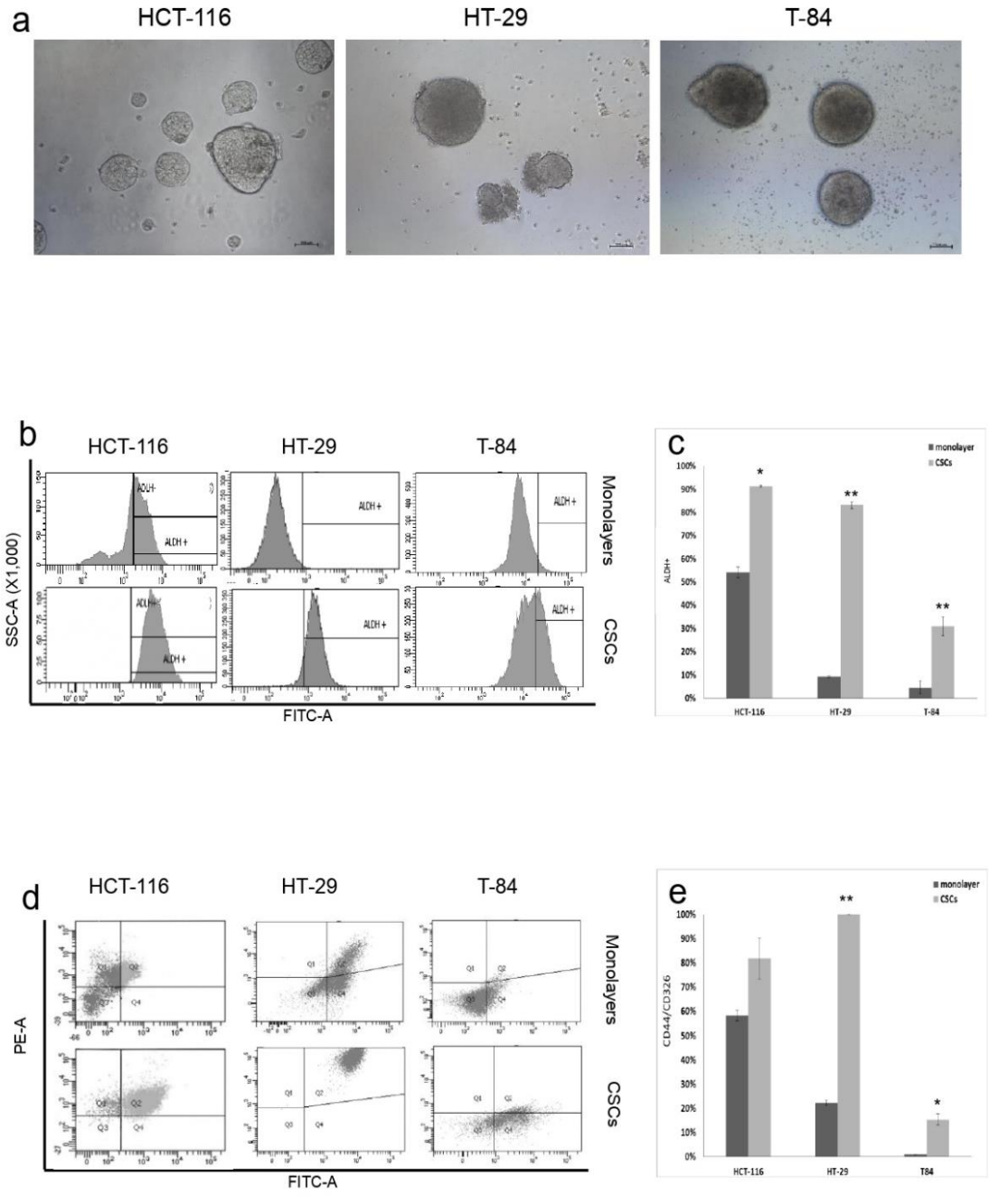


*Figure 15. CSC enrichment of HCT-116, HT-29 ad T-84 cells and flow cytometric analyses of ALDH1 and CD44/CD326. (A) Secondary colonospheres enriched in CSCs after 6 days of cultures; bar = 100 µm. (B) Flow citometry histograms of ALDH1 activity in CSCs and monolayers; x-axis: FITC-A/ALDH1, y-axis: SSC-A/side scatter. (C) Statistical analysis of ALDH1 activity in CSCs ad monolayers; * p < 0.05, **p < 0.01. (D) Representative flow cytometry cytogram plot of CD44/CD326 ratio in CSCs ad monolayers x-axis: FITC-A/CD326, y-axis: PE-A/CD44, Q1: CD44+/CD326-, Q2 CD44+/CD326+, Q3: CD44-/CD326-, Q4 CD44-/CD326+. (E) Statistical analysis of CD44/CD326 in CSCs and monolayers; *p < 0.05, **p < 0.01. Source* (Farace et al., 2020)

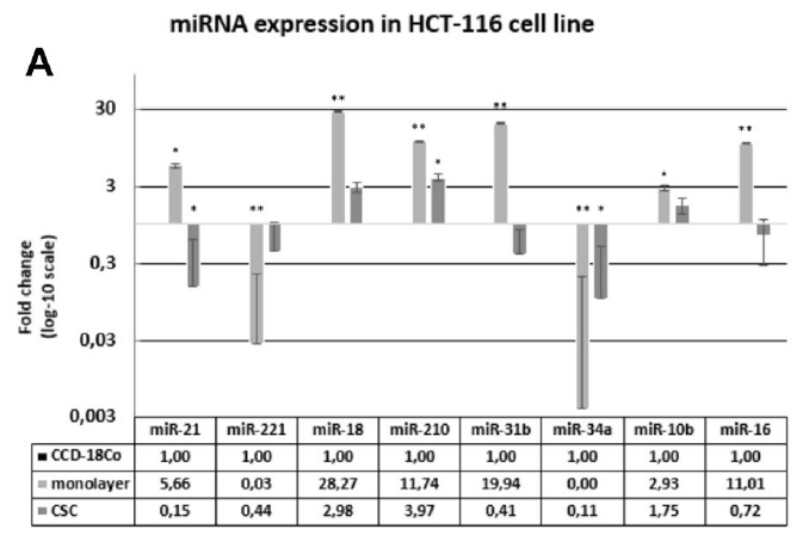
8.1.2. miRNA expression in cell lines: comparison between CSCs and monolayer

The set of miRNAs selected from literature was evaluated by an *in vitro* model that allows distinguishing cancer-related miRNAs (both stem and non stem) from miRNAs of non

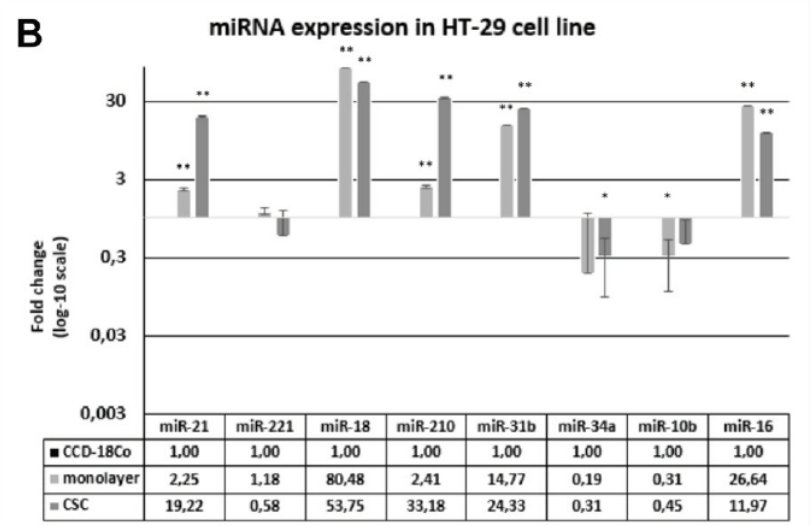
tumoral cells. The Ct values of the miRNAs in the three CSCs and the corresponding monolayer cells were normalized to the miRNA contents of the CCD-18Co cell line, which was included in the study as a healthy non tumoral model. In HCT-116, the fold change (FC) values for miR-18a ($p < 0.05$ for monolayer), miR-210 ($p < 0.05$ both in CSC and monolayer) and miR-10b ($p < 0.05$ for monolayer) were highest in CSC and/or monolayer, whereas the FC values of miR-221 ($p < 0.05$ for monolayer) and miR-34a ($p < 0.05$ both in CSC and monolayer) were highest in the CCD-18Co cells. On the contrary, miR-21 was most expressed in the HCT-116 monolayer and lower in the CSC than in CCD-18Co ($p < 0.05$ for both CSC and monolayer). Similar patterns were also observed for miR- 31b and miR-16, but were only statistically significant in the monolayer (Figure 16A). In the HT-29 cell line, miR-21, miR-18a, miR-210, miR-31b and miR-16 were higher in the CSC and monolayer than in the healthy control cell line ($p < 0.05$), whereas miR-34a and miR-10b were higher in the CCD-18Co cells than in the CSC or monolayer of HT-29 ($p < 0.05$). miR-221 was downregulated in the HT-29 CSC and upregulated in the monolayer relative to its level in healthy cells, but the differences were not statistically significant (Figure 16B). In the T-84 model, miR-10b ($p < 0.05$ in CSC and monolayer) and miR-210 ($p < 0.05$ in CSC) were higher in the CSC and/or monolayer than in CCD-18Co, whereas miR-34a showed the contrary behaviour ($p < 0.05$ for both CSC and monolayer of T-84). miR-221 was significantly lower in T-84 monolayer than in healthy cells, whereas miR-16 was higher in the T-84 monolayer but lower in the CSC than in healthy cells, although the differences were not statistically significant (Figure 16C).

Then the date were analysed from another perspective, in order to have global vision of the miRNA expression in the monolayer and CSC conditions. To this end we calculate the average Ct values of the three lines in each conditions, monolayer and CSCs, then we calculate the FC between these and the control CCD-18Co. miR-21 ($p = 0.03$ for monolayers, $p = 0.02$ for CSCs), miR-18a ($p = 0.001$ for monolayers, $p = 0.01$ for CSCs),

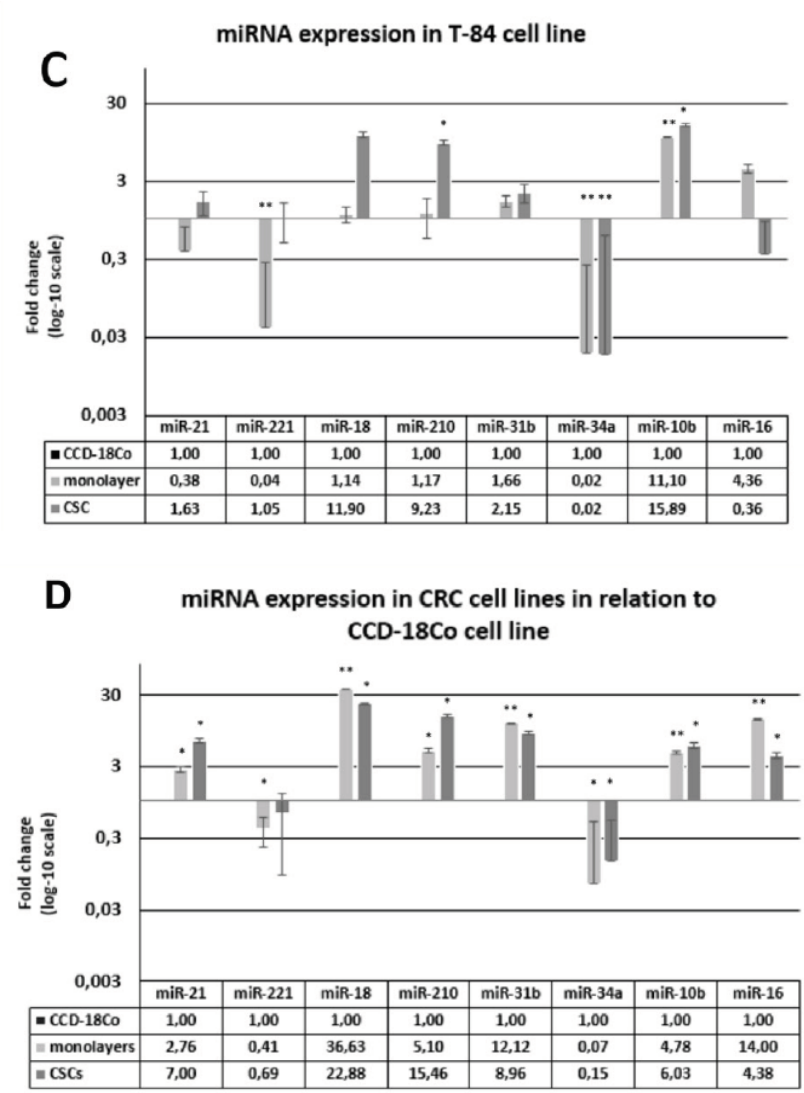
miR-210 (p = 0.01 for monolayers, p = 0.01 for CSCs), miR-31b (p = 0.01 for monolayers, p = 0.02 for CSCs), miR-10b (p = 0.01 for monolayers, p = 0.03 for CSCs) and miR- 16 (p = 0.007 for monolayers, p = 0.038 for CSCs) were globally associated with the cancer phenotype, whereas miR-221 (p = 0.019 for monolayers, p > 0.05 for CSCs) and miR-34a (p = 0.01 for monolayers, p = 0.02 for CSCs) were usually associated with the healthy phenotype (Figure 16D). Details about the results showed in tables under the images.



	miR-21	miR-221	miR-18	miR-210	miR-31b	miR-34a	miR-10b	miR-16
CCD-18Co	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00
monolayer	5,66	0,03	28,27	11,74	19,94	0,00	2,93	11,01
CSC	0,15	0,44	2,98	3,97	0,41	0,11	1,75	0,72



	miR-21	miR-221	miR-18	miR-210	miR-31b	miR-34a	miR-10b	miR-16
CCD-18Co	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00
monolayer	2,25	1,18	80,48	2,41	14,77	0,19	0,31	26,64
CSC	19,22	0,58	53,75	33,18	24,33	0,31	0,45	11,97



	miR-21	miR-221	miR-18	miR-210	miR-31b	miR-34a	miR-10b	miR-16
CCD-18Co	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00
monolayer	0,38	0,04	1,14	1,17	1,66	0,02	11,10	4,36
CSC	1,63	1,05	11,90	9,23	2,15	0,02	15,89	0,36

	miR-21	miR-221	miR-18	miR-210	miR-31b	miR-34a	miR-10b	miR-16
CCD-18Co	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00
monolayers	2,76	0,41	36,63	5,10	12,12	0,07	4,78	14,00
CSCs	7,00	0,69	22,88	15,46	8,96	0,15	6,03	4,38

Figure 156. Expression of miRNAs in HCT-116, HT-29 and T-84 CSCs and monolayers, in relation to the expression in ***CCD-18Co cells.*** *(A) miRNAs expression in HCT-116 CSC and monolayer normalized on CCD-18CO. (B) miRNAs expression in HT- 29 CSC and monolayer normalized on CCD-18CO. (C) miRNAs expression in T-84 CSC and monolayer normalized on CCD-18CO. (D) Average of miRNAs expression in CSCs and monolayer normalized on CCD-18CO. * p < 0.05, **p < 0.01. Source* (Farace et al., 2020).

8.1.3. Hoechst 33342 analysis

For all the three cell lines, HT-29, T-84, and HCT-116 we observe the values relative to the positivity to Hoechst 33342 of the cultured cells in adhesion (monolayer) and of the cultured cells in suspension (CSCs). By positivity to Hoechst, we mean the amount of dye (and therefore of the drug) that has been retained by the cells. In HT-29 we registered a value of 90.65 for the monolayer, and of 31.15 for the CSCs (Figure 17A); In T-84 we registered a value of 96 for the monolayer, and of 8.1 for the CSCs (Figure 17B); In HCT-

116 we registered a value of 86 for the monolayer, and of 46.85 for the CSCs (Figure 17C). In all the three cell models, the comparison between monolayer and CSCs was supported by a p value lower than 0.01. As can be seen from the representation, the values in monolayers were clearly higher than the values of CSCs.

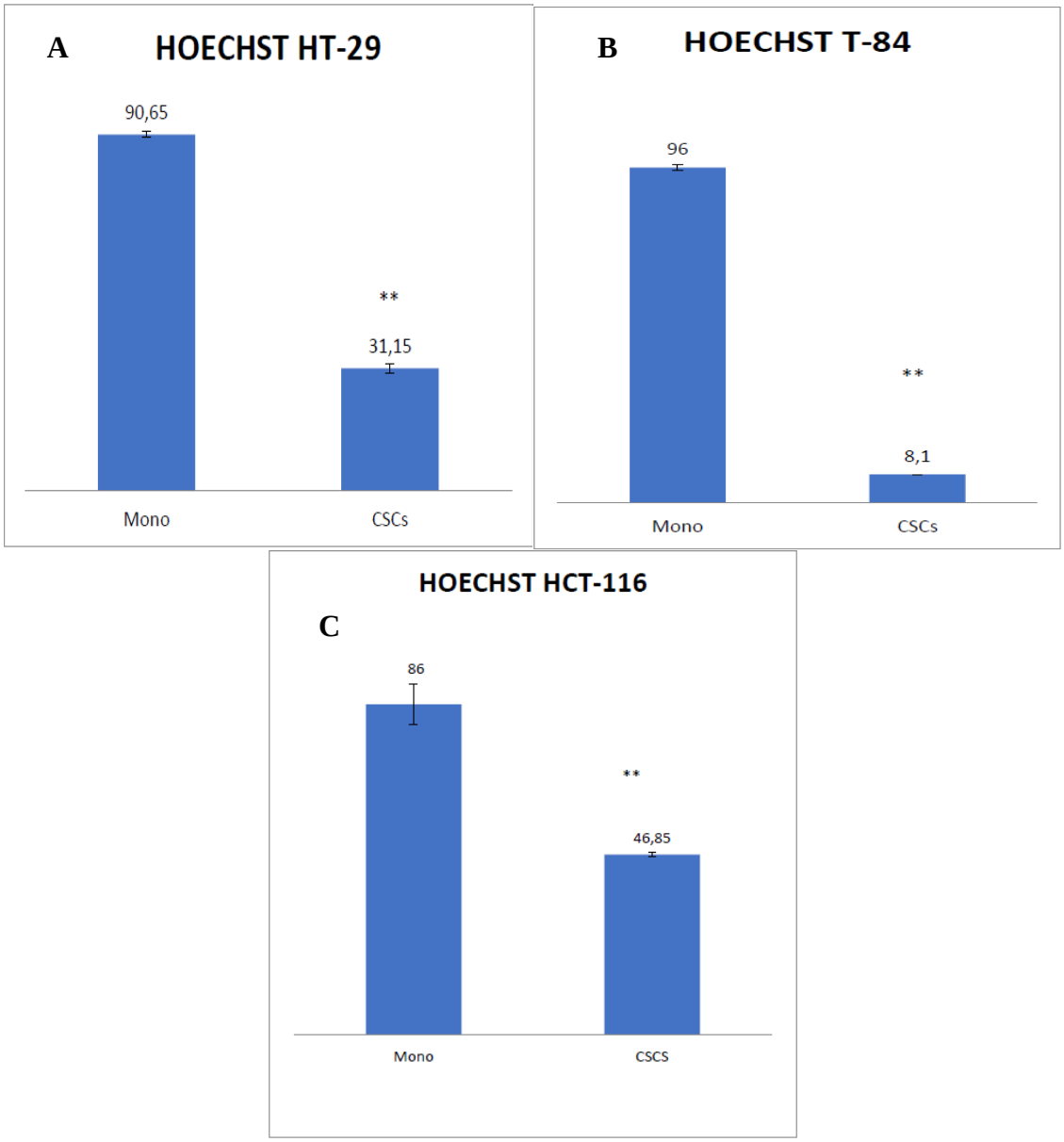
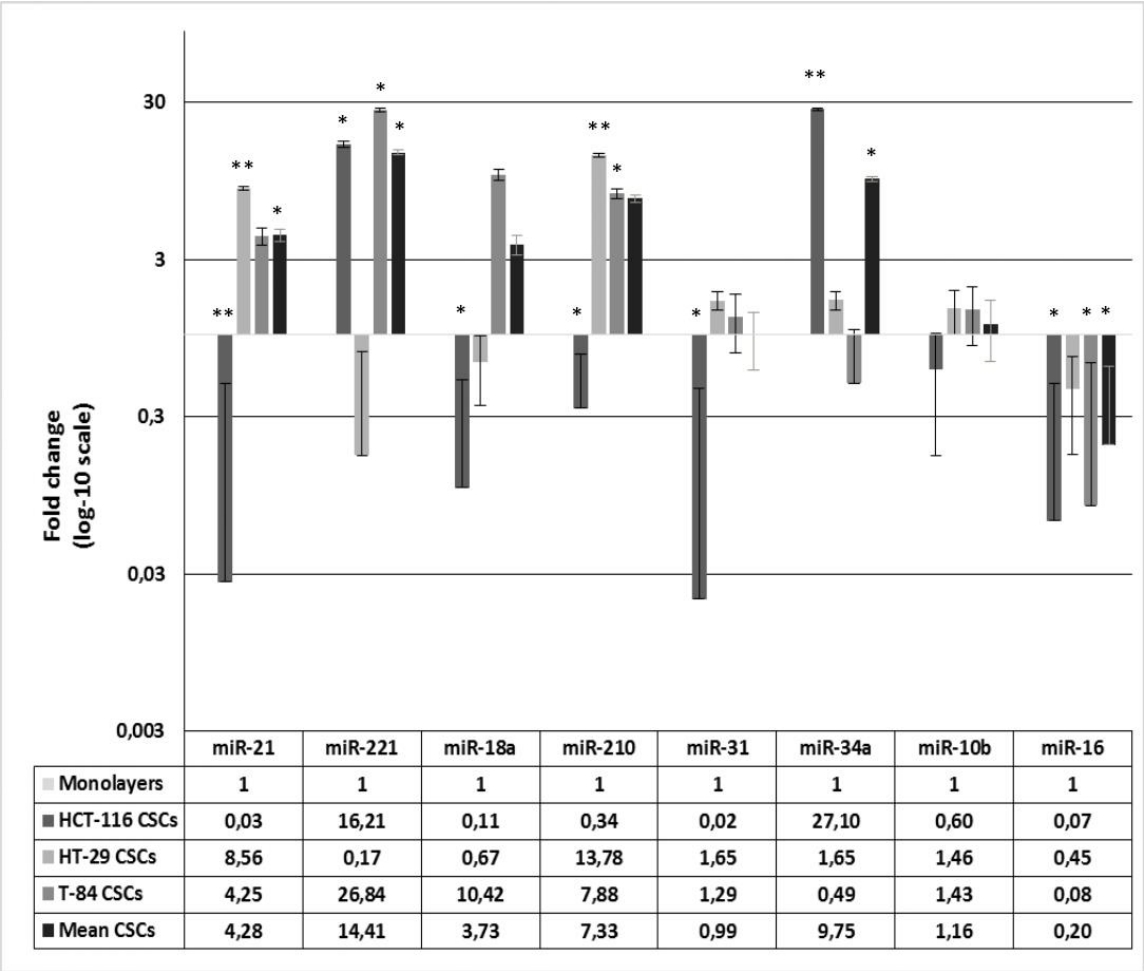


Figure 167. Hoechst 33342 analysis comparing the monolayers vs CSC; a) HT-29, b) T-84 and c) HCT-116

8.1.3.1. miRNA expression in colorectal CSC and monolayer cell lines

To better understand the miRNA pattern implicated in the CSC phenotype, the comparison between the expression of the selected miRNA in monolayer and CSC phenotype was carried out in the three cell lines and in the average of the three cell lines

for monolayer and for CSC, as showed in Figure 18. The highly expressed miRNA in HCT-116 CSC, were miR-34a (FC = 27.09; p = 0.0071) and miR-221 (FC = 16.20; p = 0.0231), whereas all the other miRNAs analyzed were downregulated (miR-21, miR-18a, miR-31, miR-16; all $p < 0.05$). In the CSC derived from HT-29, the higher expression was observed for miR-210 (FC = 13.78; p = 0.0056) and miR-21 (FC = 8.55; p = 0.0047); the same behavior was observed in miR-31, miR-34a and miR-10b, although the changes were not statistically significant ($p > 0.05$). Similarly, miR-221, miR-18a and miR-16 were downregulated, although not statistically significantly, with no robust miRNA downregulation in HT-29 CSC when compared with the monolayer counterpart. T-84 CSC the most expressed miRNA were miR-221 (FC = 26.84; p = 0.0138) and miR-210 (FC = 7.87; p = 0.0374). Although miR-18a (FC = 10.42; p = 0.0575) and miR-21 (FC = 4.24; p = 0.0618) showed an important FCs, these were not supported by statistical analyses. The uniquely downregulated miRNA in T-84 CSCs was miR-16 (FC = 0.08; p = 0.0245), whereas the differences in miR-31, miR-34a and miR-10b expression between the T-84 CSC and monolayer cells were not statistical significant. When the data from all three CSC models were merged together (Figure 3), the CSCs-related miRNAs most expressed were miR-21 (FC = 4.27; p = 0.0355), miR-221 (FC = 14.40; p = 0.0233), miR-210 (FC = 7.33; p = 0.0216) and miR-34a (FC = 9.74; p = 0.0146), whereas miR-18a, miR-31 and miR-10b showed similar expression to that in the three monolayers. Interestingly, miR-16 (FC = 0.19; p = 0.0319) was the only significantly downregulated miRNA in all three CSC models, considered alone or together (no expression), whereas it showed the basal level of expression in the monolayer cells.



	miR-21	miR-221	miR-18a	miR-210	miR-31	miR-34a	miR-10b	miR-16
Monolayers	1	1	1	1	1	1	1	1
HCT-116 CSCs	0,03	16,21	0,11	0,34	0,02	27,10	0,60	0,07
HT-29 CSCs	8,56	0,17	0,67	13,78	1,65	1,65	1,46	0,45
T-84 CSCs	4,25	26,84	10,42	7,88	1,29	0,49	1,43	0,08
Mean CSCs	4,28	14,41	3,73	7,33	0,99	9,75	1,16	0,20

Figure 18. ***Expression of miRNAs in HCT-116, HT-29, T-84 CSCs in relation to the expression in respective monolayers.*** *Data are shown for CSC separately and as average of miRNAs expression in CSCs normalized to monolayers * $p < 0.05$, ** $p < 0.01$.*

8.2. Patients

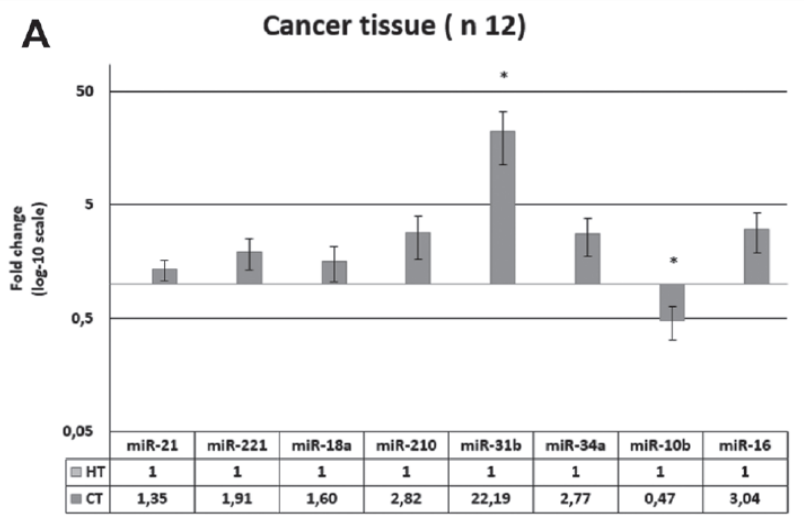
8.2.1. miRNA expression in cancer tissues and sera from CRC patients

From the comparison between cancer tissue and healthy tissue, the hyperexpression of all the miRNA analyzed emerged, with the only exception for miR-10b. Furthermore, the unique statistically significant was observed to for miR-31b, which was highly upregulated (FC = 22.19; p = 0.0019), and for miR-10b, which was significantly downregulated (FC = 0.47; p = 0.0088) (Figure 19A). Then, the patients were divided into two categories according to their tumour stage (LOW: stages 0–IIIA, n = 5; or HIGH: stages IIIB–IVA, n = 7). Comparing cancer tissue *vs* healthy tissue, the upregulation of miR-31b and the downregulation of miR-10b was observed, significantly more often in HIGH patients and

in LOW patients, respectively. Other interesting data emerged from this division, was the acquiring of the significance of miR-16 in LOW group (FC = 3.83; p = 0.0025), significance not observed in the total group of patients or in the HIGH group (Figure 19B). When the patients were grouped considering the presence or absence of metastasis (NO, n = 7; YES, n = 5), a similar pattern was observed, with the exception for miR-31b, that was also significantly upregulated in no-Met patients ($p < 0.05$; Figure 19C).

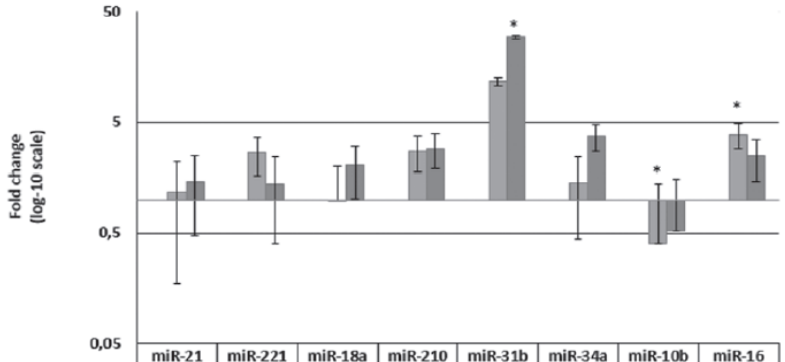
The miRNA analysis in serum, produced results in arbitrary units (a.u.) for each miRNA. It showed that miR-221 (a. u. = 3.72; p = 0.0046) and miR-16 (a. u. = 11.11; p = 0.003) were upregulated and that miR-34a (a. u. = 0.17; p = 0.0015) and miR-10b (a. u. = 0.44; p = 0.0277) were downregulated in serum from the CRC patients relative to their expression in the corresponding healthy tissue (Figure 19D). Both miR-18a and miR- 31b were upregulated and miR-21 was downregulated in serum, but with not statistically significant. After the patients were divided in two groups according to tumour stage (LOW, n = 5; HIGH, n = 7), the serum–healthy tissue analysis showed significant upregulation of miR-221 and miR-21 and significant downregulation of miR-34a in the LOW and HIGH groups of patients relative to the controls, and miR-16 upregulation (a. u. = 8.35; p = 0.0002) and miR-10b downregulation (a. u. = 0.42; p = 0.0077) in particular in the LOW patient group (Figure 19E). When the patients were grouped by metastasis (non metastatic = 7; metastatic = 5), in metastatic patients, just miR-21 (a. u. = 2.02; p = 0.0156) and miR-18a (a. u. = 2.92; p = 0.0175) were significantly higher in serum than in healthy tissue, whereas in non metastatic patients, miR-21 (a. u. = 0.18; p = 0.0001), miR-210 (a. u. = 0.46; p = 0.0127), miR-34a (a. u. = 0.11; p = 0.0001) and miR-10b (a. u. = 0.37; p = 0.0072) were lower in serum than in healthy tissue, and both miR-221 (a. u. = 4.31; p = 0.0023) and miR-16 (a. u. = 8.26; p = 0.0001) were higher in serum than in healthy tissue (Figure 19F). Moreover, the miR-18 levels in serum were higher in metastatic patients than in the non metastatic patients (a. u = 3.43; p = 0.0059; Figure 19F).

Doing CT–S comparison the follow results were obtained: miR-31b expression was significantly higher in cancer tissue than in serum in the whole CRC patients group, and was also significantly higher in cancer tissue than in serum in the LOW (p = 0.0265), HIGH (p = 0.0056), non metastatic (p = 0.0021) and metastatic (p = 0.0339) patients taken alone. According to TNM group division mentioned before in this paragraph, higher miR-21 (p = 0.0204), miR-31b (p = 0.0265) and miR-34a (p = 0.003) where higher in cancer tissue than in serum in the LOW patients, whereas no other differences were observed in the HIGH patients. In relation to metastasis, only miR-31b (p = 0.0399) was higher in cancer tissue than in serum in metastatic patients, whereas miR-21 (p = 0.0007), miR-210 (p = 0.0334), miR-31b (p = 0.0021) and miR-34a (p = 0.0001) where higher in cancer tissue than in serum in the non metastatic patients. This differed from the pattern for miR-16, whose expression was higher in serum than in cancer tissue in non metastatic patients (p = 0.0275).



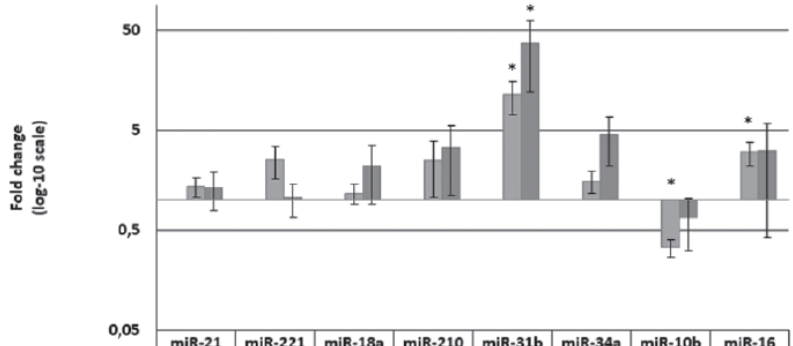
	miR-21	miR-221	miR-18a	miR-210	miR-31b	miR-34a	miR-10b	miR-16
HT	1	1	1	1	1	1	1	1
CT	1,35	1,91	1,60	2,82	22,19	2,77	0,47	3,04

B **Cancer tissue by grading (n 5 vs 7)**



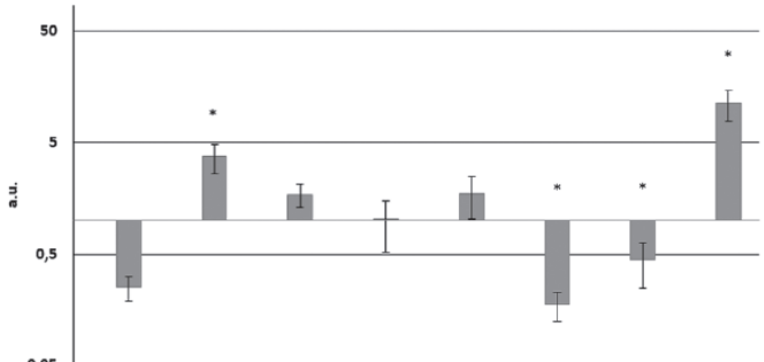
	miR-21	miR-221	miR-18a	miR-210	miR-31b	miR-34a	miR-10b	miR-16
HT (n=12)	1	1	1	1	1	1	1	1
CT LOW (n=5)	1,17	2,62	1,00	2,74	11,73	1,44	0,40	3,83
CT HIGH (n=7)	1,47	1,40	2,02	2,88	29,66	3,73	0,53	2,47

C **Cancer tissue by metastasis (n 7 vs 5)**

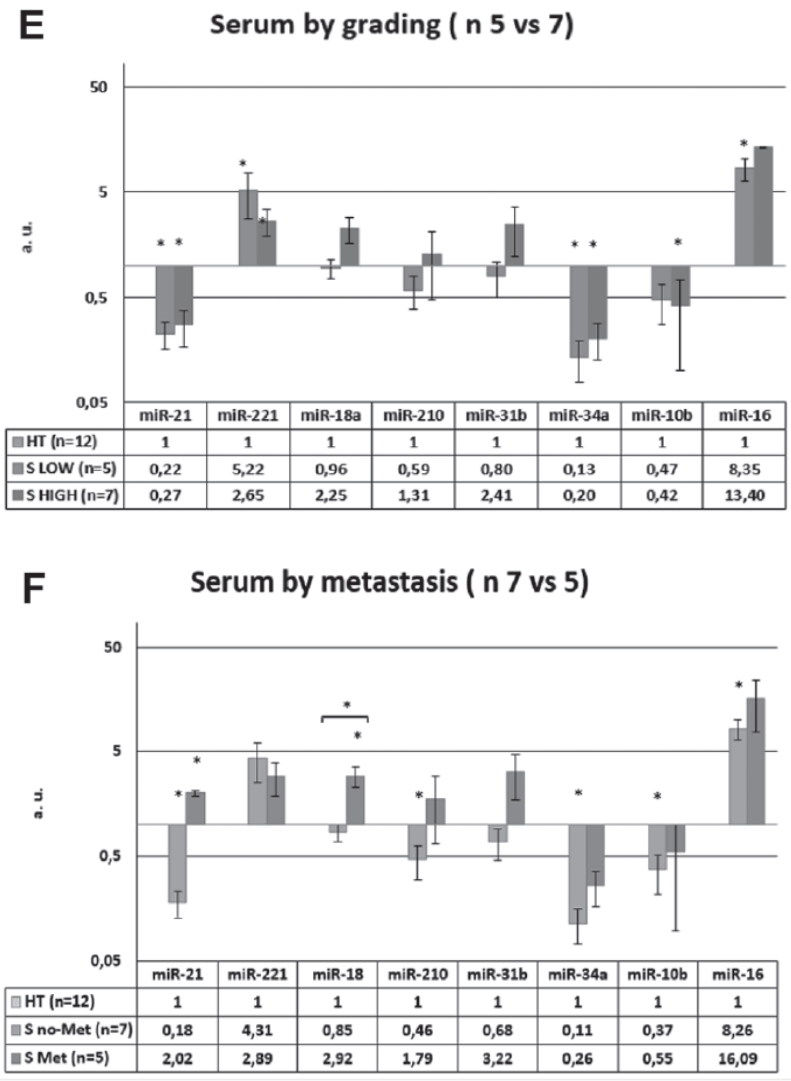


	miR-21	miR-221	miR-18a	miR-210	miR-31b	miR-34a	miR-10b	miR-16
HT (n=12)	1	1	1	1	1	1	1	1
CT no-Met (n=7)	1,36	2,53	1,16	2,48	11,37	1,55	0,33	2,99
CT Met (n=5)	1,32	1,05	2,21	3,30	37,33	4,49	0,68	3,11

D **Serum (n 12)**



	miR-21	miR-221	miR-18	miR-210	miR-31b	miR-34a	miR-10b	miR-16
HT	1	1	1	1	1	1	1	1
S	0,25	3,72	1,71	1,01	1,74	0,17	0,44	11,11



	miR-21	miR-221	miR-18a	miR-210	miR-31b	miR-34a	miR-10b	miR-16
HT (n=12)	1	1	1	1	1	1	1	1
S LOW (n=5)	0,22	5,22	0,96	0,59	0,80	0,13	0,47	8,35
S HIGH (n=7)	0,27	2,65	2,25	1,31	2,41	0,20	0,42	13,40

	miR-21	miR-221	miR-18	miR-210	miR-31b	miR-34a	miR-10b	miR-16
HT (n=12)	1	1	1	1	1	1	1	1
S no-Met (n=7)	0,18	4,31	0,85	0,46	0,68	0,11	0,37	8,26
S Met (n=5)	2,02	2,89	2,92	1,79	3,22	0,26	0,55	16,09

*Figure 179. Expression of miRNAs in cancer tissue (CT) and serum (S) of CRC patients. The data of CT ad S were both normalized to healthy tissue in each patients. (A) miRNAs level in CT vs HT of 12 patients. (B) miRNAs level in CT vs HT in patients divided in two groups in function of patients' grade. (C) miRNAs level in CT vs HT in patients divided in two groups in function of metastasis. (D) miRNAs level in S vs HT of 12 patients. (E) miRNAs level in S vs HT in patients divided in two groups in function of patients' grade. (F) miRNAs level in S vs HT in patients divided in two groups in function of metastasis. * p < 0.05, **p < 0.01.*

8.2.2. Spearman's correlation analysis of miRNAs and clinical parameters

Spearman's correlation matrices between the miRNA levels in cancer tissue or serum and clinical parameters identified a set of significant positive and negative correlations. In cancer tissue, miR-221 correlated positively with sex and negatively with aspartate aminotransferase (AST); in serum, miR-221 correlated positively with age and negatively with both alanine aminotransferase (ALT) and (AST). In cancer tissue, miR-18a correlated positively with low-density lipoprotein (LDL) and negatively with age; in serum, miR-18a correlated positively with metastasis and triglycerides. In cancer tissue, miR-34a

correlated positively with alkaline phosphatase (ALP; Table 2). The correlations between the different variables and miR-21, miR-210, miR-31b, miR-10b and miR-16 (cancer tissue or serum) and miR-34a (serum) were not significant (not shown).

Table 2 Spearman's rank correlation coefficients between miRNA levels in patient's cancer tissues (CT) or serum (S) and demographic/clinical variables

	miR-221		miR-18a		miR-34a
	CT	S	CT	S	CT
Age	–0,0866	0,6879*	–0,6651**	0,4009	0,3554
Sex	0,7769*	0,5379	0,0000	–0,3586	–0,3586
Met	–0,4781	–0,3586	0,1793	0,6574*	0,0598
GGT1	–0,5818	–0,4636	0,2091	0,4818	0,3091
ALT/GPT	–0,5138	–0,6055**	0,0459	–0,1560	0,0183
AST	–0,7002**	–0,6362**	0,1098	0,3341	0,1327
CPK	–0,0091	–0,3455	-0,0364	–0,0818	–0,1455
ALP	–0,4000	0,0455	-0,2364	0,4364	0,6091*
CHOL	0,5000	–0,0636	0,5545	–0,2545	–0,4455
HDL	0,1727	0,2182	0,0182	–0,5818	–0,4000
LDL	0,5057	–0,3462	0,6743*	–0,1321	–0,4191
TG	–0,2636	–0,0091	0,2182	0,6455*	–0,0091
GLY	–0,3319	–0,0935	–0,0795	0,1122	–0,4207

8.3. Results section 2

8.3.1. Serum Small RNA-Sequencing

The RNA-sequencing of the pooled serum RNA samples, showed differential expression levels of miR-486-5p, with a progressive reduction of expression with the disease progression. The higher expression was observed in the non-tumor control group (average number of copies 1,364,721, standard error 57,572), and the lowest in the metastatic group (average number of copies 752,483, standard error 61,730) which includes patients with both lymphatic and distant metastasis. We observed statistically significant differences between the metastatic and non-tumor groups (log2 FC = −0.85, p = 0.0008; FDR-adjusted p = 0.03), while the tumor and non-tumor groups (log2 FC = −0.63, p = 0.007, FDR-adjusted p > 0.05), and the metastatic and tumor groups (log2 FC = −0.22, p = 0.03; FDR-adjusted p > 0.05) were not supported by statistically significance. Calculating the difference among the three groups with ANOVA, a p-value was 0.008 was obtained. The expression of hsa-miR-342-3p, used as the reference miRNA for normalisation of miR-486-5p, was similar in all the three groups: non-tumor (average number of copies 3069,

standard error 147), tumor (average number of copies 3074, standard error 24), and metastatic (average number of copies 3074, standard error 574) (Figure 20). Table 3 shows the data of the most significantly dysregulated miRNAs.

Table 3 miR-Seq: average number of copies, p and adjusted p of most significantly dysregulated miRNAs.

miRNA	pvalue	padj	Metastatic_1	Metastatic_2	Tumor_1	Tumor_2	Healthy_1	Healthy_2
hsa-miR-3614-5p	7.57797×10^{-9}	2.50831×10^{-6}	588.15	602.64	0	0	0	0
hsa-miR-1247-5p	4.44313×10^{-8}	3.91482×10^{-6}	923.17	124.99	0	0	0	0
hsa-miR-26b-3p	2.49849×10^{-8}	3.91482×10^{-6}	405.75	0	693.60	0	0	0
hsa-miR-370-3p	4.7309×10^{-8}	3.91482×10^{-6}	930.61	138.38	0	0	0	0
hsa-miR-371b-5p	3.02639×10^{-7}	2.00347×10^{-5}	655.15	196.42	0	0	0	0
hsa-miR-92a-1-5p	1.18488×10^{-6}	6.53656×10^{-5}	201.01	620.50	0	0	0	0
hsa-miR-1343-3p	1.58942×10^{-6}	7.51568×10^{-5}	0	178.56	0	449.91	0	0
hsa-let-7b-3p	1.87635×10^{-6}	7.76339×10^{-5}	450.42	111.60	0	0	0	0
hsa-miR-3130-5p	3.90736×10^{-6}	0.000143704	171.23	0	610.03	0	0	0
hsa-miR-93-5p	3.5912×10^{-5}	0.001188687	23101.53	21016.67	18685.45	26506.86	47283.15	57832.90
hsa-miR-223-3p	0.000104139	0.003133635	355620.64	417538.85	288671.90	294471.36	175026.35	162243.02
hsa-miR-431-5p	0.000641093	0.017683487	0	0	0	0	660.62	748.75
hsa-miR-320a	0.00126751	0.032272748	129310.50	120542.28	57794.55	53603.04	191473.93	241138.63
hsa-miR-486-5p	0.001484273	0.03407904	708832.85	796133.48	937832.71	815698.98	1422293.08	1307148.95
hsa-miR-628-3p	0.001544367	0.03407904	416.91	437.47	434.54	183.78	0	0
hsa-miR-7-1-3p	0.002010025	0.041582387	0	441.94	626.75	1184.98	0	0

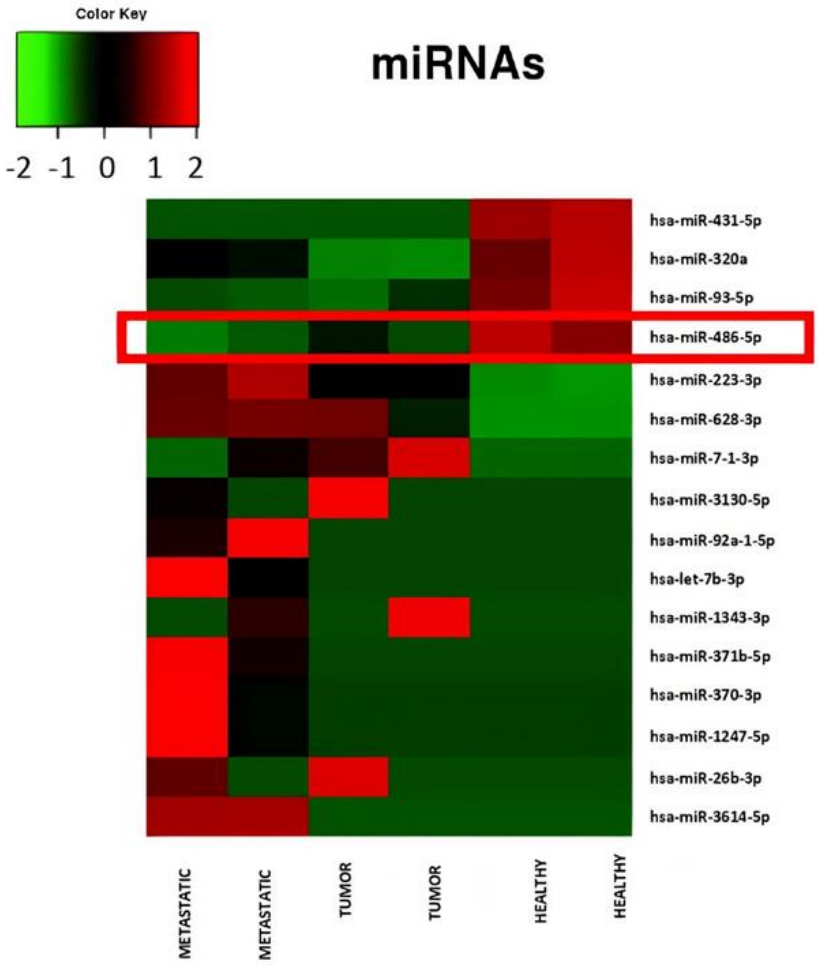


Figure 20. Heatmap relative to miRNA expression levels measured by miR-Seq and significantly altered in serum pools from patients in different condition: controls, patients with low stage CRC (stage I to II), patients with advanced stage

CRC (stage III to IV). A progressive down-regulation of miR-486-5p from controls to advanced stage CRC patients is showed. Source: (Pisano et al., 2020)

8.3.2. miR-486-5p expression in sera determined with real-time RT-PCR

We confirmed the trend previously reported in the tumor vs. non-tumor group comparison (FC = 2.2, $p > 0.05$) and in the metastatic vs. non-tumor group comparison (FC = 0.89, $p > 0.05$), when the average expression of hsa-miR-342-3p, hsa-223-3p, and hsa-miR-93-5p was used as reference for normalisation. Furthermore, we confirmed the statistically significant differences observed between tumor and metastatic groups (FC = 2.47, $p = 0.02$) (Figure 21A). When hsa-miR-342-3p was used as reference miRNA, we observed a differential expression of miR-486-5p in the tumor (FC = 2.73, $p > 0.05$) and metastatic CRC groups (FC = 0.79, $p > 0.05$) with respect to controls. There was also a statistically significant difference between tumor and metastatic groups (FC = 3.45; $p = 0.02$; Figure 21B). Then the control group was compared also with the total tumor group (stages I–IV), and an up-regulation of miR-486-5p in CRC patients was observed, using as reference the average of hsa-miR-342-3p, hsa-223-3p, and hsa-miR-93-5p (Figure 21B, FC = 1.35), or the hsa-miR-342-3p alone (Figure 21C, FC = 1.47).

8.3.3. miR-486-5p levels in stool by small RNA-sequencing

In the stools of the CRC patients, we found overexpression of miR-486-5p when compared with healthy controls. The average number of reads were 43.65 in the controls and 223.67 in the CRC patients, with log2 FC = 2.36 and adjusted $p = 3.6 \times 10{-}11$ (Figure 21E).

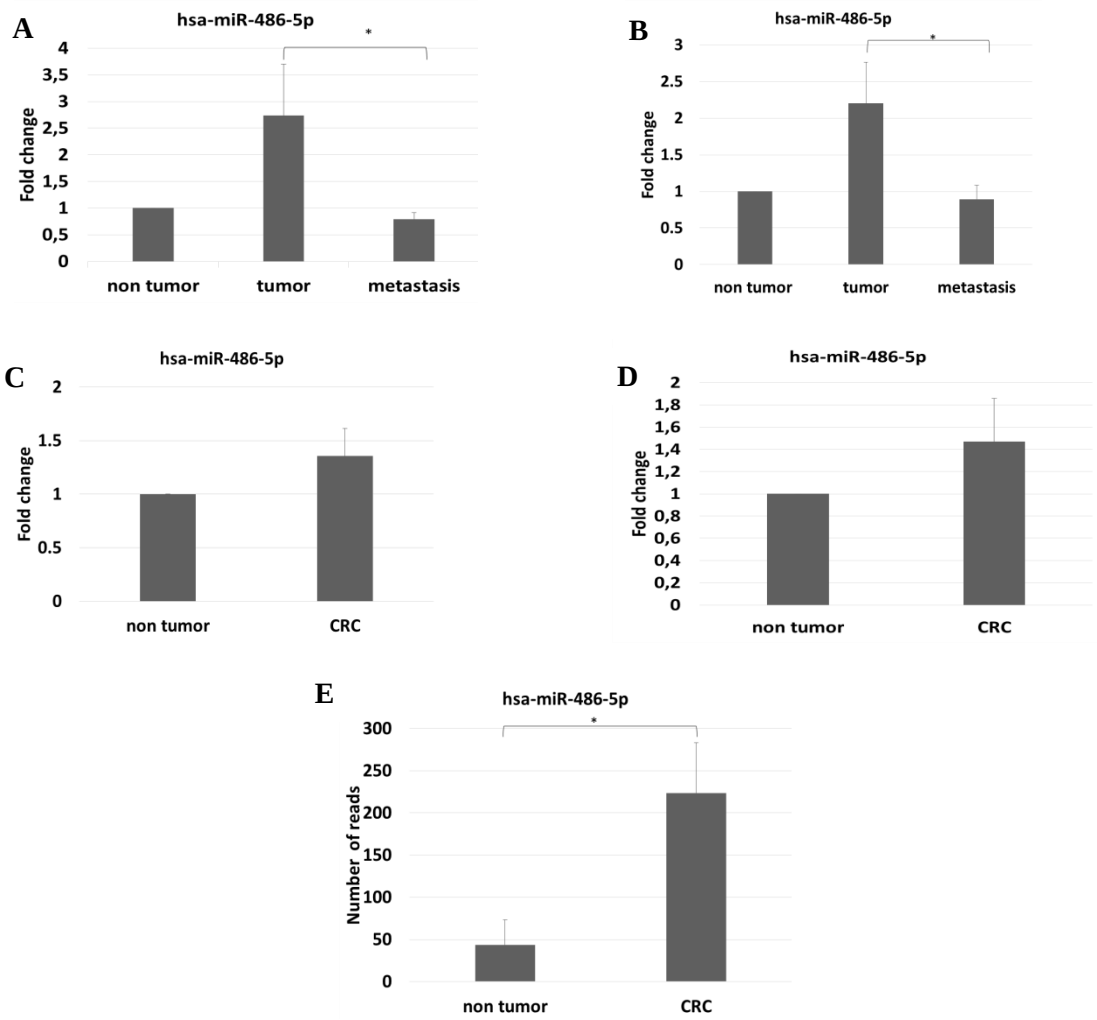


*Figure 21. **A** and **B**. miR-486-5p expression levels among the three groups analyzed, measured by Real-time RT-PCR in serum. **A.** Normalization with the mean expression levels of hsa-miR-342-3p, hsa-miR-223-3p and hsa-miR-93-5p; **B.** Normalization done by using has-miR-342-3p. **C** and **D.** miR-486-5p expression levels by comparing control and tumor group, as measured by Real-time RT-PCR in serum. **C.** Normalization with the mean expression levels of hsa-miR-342-3p, hsa-miR-223-3p and hsa-miR-93-5p; **D.** Normalization don by using miR-342-3p. **E.** Stool miR-486-5p expression levels as measured by small RNA-sequencing, in controls and CRC patients. The graphics report average values with standard errors. The symbol * indicates differences supported by statistical significance.*

8.3.4. Meta-Analysis

From the meta-analysis of the expression levels of miR-486-5p in serum and tissue samples from CRC patients and healthy controls were used the datasets from the GEO database. In serum, was observed a standardized difference between cases and controls of 1.01 (95% confidence interval 0.70; 1.32) (Figure 22A). In tissue samples, was observed a standardized difference between cases and controls risk ratio of −0.61 (95% confidence interval −0.70; −0.52) (Figure 22B). These data indicate that miR-486-5p was

overexpressed in the sera of patients relative to the controls, whereas it was down-regulated in tumor tissues in comparison with healthy tissues. The dataset GSE39845, allowed to us observe a difference between early-stage (stage I–II) and late-stage CRC (stage III–IV), with a progressively down-regulation with the disease progression (Figure 22C), although the difference was not statistically significant.

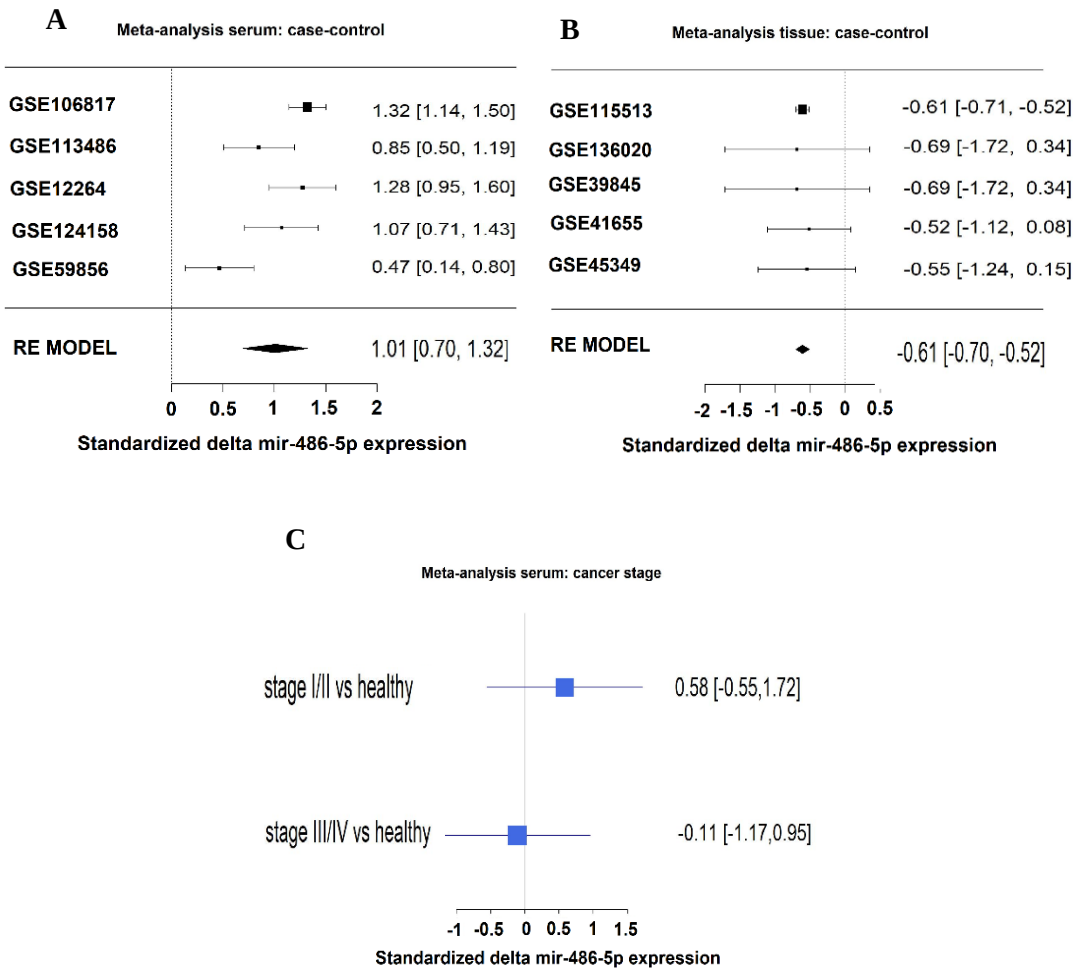


*Figure 22. (**A**) Meta-analysis of miR-486-5p levels in serum by the use of five datasets. Higher levels of miR-486-5p were observed in the case groups; (**B**) meta-analysis of miR-486-5p levels in tissues by the use of five datasets. Lower levels of miR-486-5p were observed in the case group; (**C**) expression levels of miR-486-5p related to stage in GSE39845 dataset from the GEO Database. The patients were divided in two groups: low stage (stage I and II) and advanced stage (stage III and IV).*

8.3.5. Evauation of the diagnostic and prognostic power of miR-486-5p

We performed a ROC-curve analysis to investigate whether the inclusion of mir486-5p expression values, measured in sera, could increase the diagnostic power of a predictive model. Specifically, we compared the ability to discriminate between CRC patients and

healthy controls of two nested logistic regression models: the first (called model 0) includes age, sex, smoking history, levels of carcinoembryonic antigen (CEA), and levels of alkaline phosphatase as predictors; the second (called model 1) including the same predictors as model 0 plus mir486-5p.

The area under the ROC curve (AUC) for model 0 was 0.67 (95% confidence interval 0.43 – 0.91, using DeLong method), whereas the AUC for model 1 was 0.74 (95% confidence interval 0.53 – 0.95, see Figure 23.

Although the difference in the prediction performance of the two models was not statistically significant, we observed a slight increase in the AUC for the model including mir486-5p as an additional predictor, suggesting its diagnostic usefulness in combination with other well-known biomarkers.

Further, we have evaluated the prognostic utility of mir486-5p measured in sera of CRC patients *via* a Cox regression model to evaluate 5-years survival after surgery. The prediction model includes age, sex, tumour stage and grade, CEA, and alkaline phosphatase as covariates. The hazard ratio (HR) comparing CRC patients within the two extreme quartiles of mir486-5p expression was 0.22 (95% confidence interval 0.03 – 2.05), suggesting that, although the difference was not statistically significant, lower levels of mir486-5p cloud be associated with a poor prognosis, even in a model which takes into account other well-known prognostic factors like tumour grade and stage and CEA. The Kaplan Meier curve, according with the stratification of CRC patients in four groups based on mir486-5p expression in sera, is displayed in Figure 24.

Although we are aware of the limitations of these statistical analyses performed on small sample size, our results suggest mir486-5p deserves further investigation to understand whether it can become a useful diagnostic and prognostic biomarker, possibly in combination with those currently used in clinical practice.

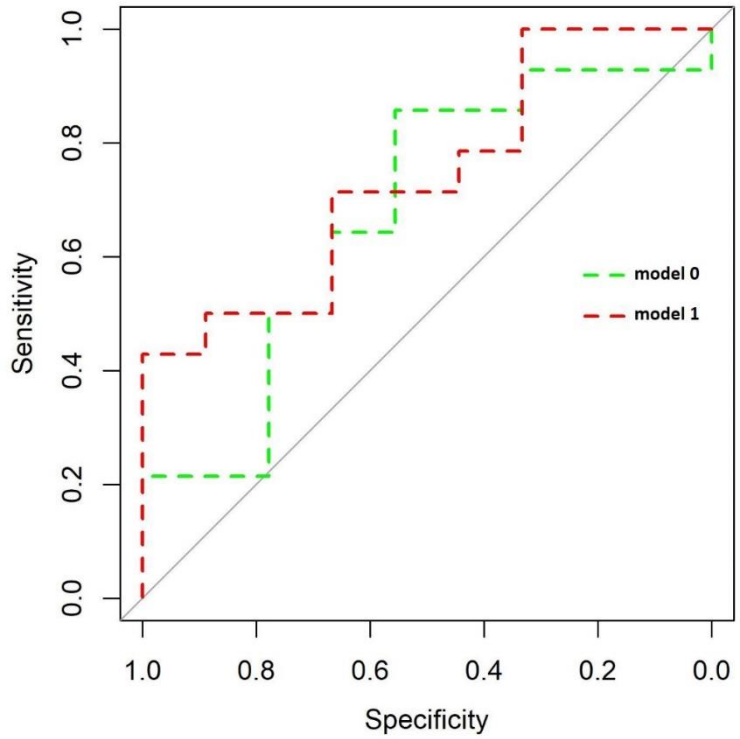


Figure 23. ROC curve of model 0 (not including mir486-5p), in green (AUC = 0.67); and ROC curve of model 1 (including mir486-5p), in red (AUC = 0.74).

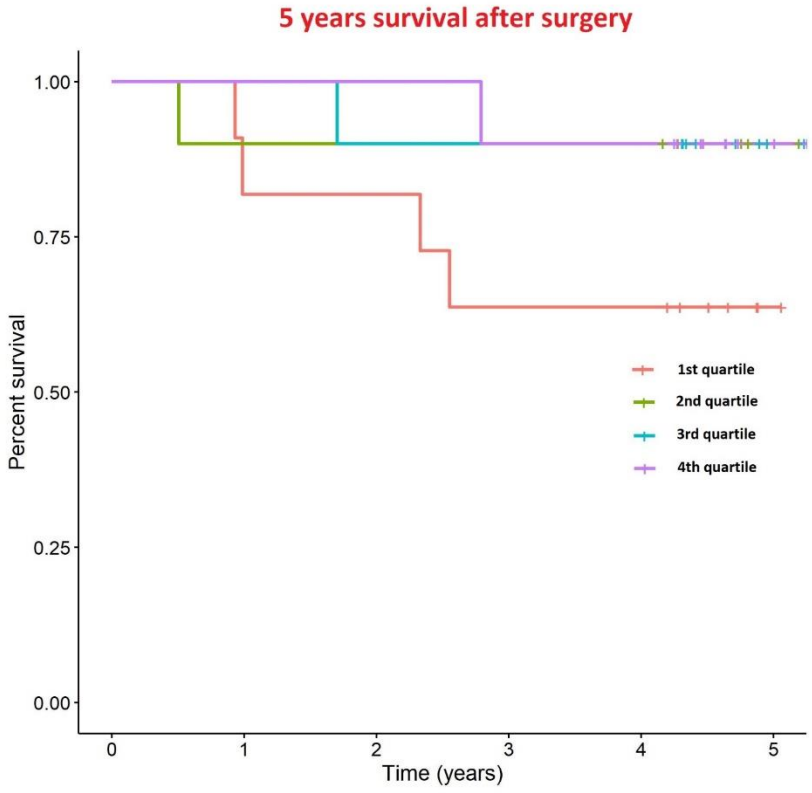


Figure 24. Kaplan Meier plot of CRC patients classified according with mir486-5p expression. Quartiles are referred to the expression of miR-486-5p in analized population.

8.3.6. miR-4865-p expression in cell lines by real-time RT-PCR analysis

The measurement of the mir-486-5p in the comparative model between monolayer and CSCs, allowed to observe a drastic decrease in the expression of this miRNA in the CSC models of all the cell lines examined (T-84, HT-29 and HCT-116). The FCs derived from this comparison are shown in Figure 25.

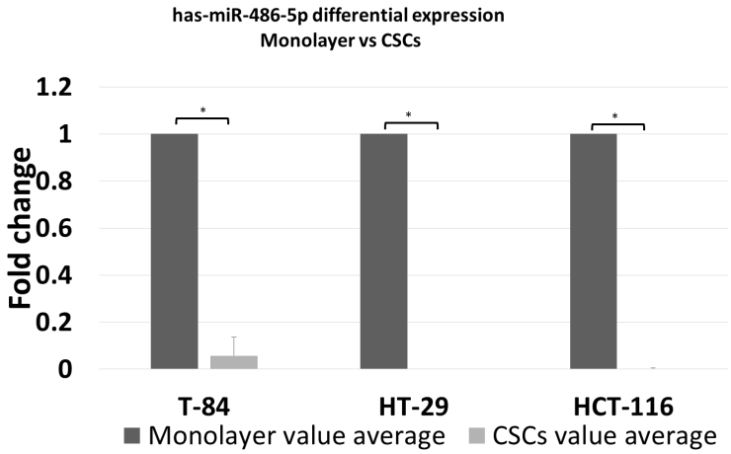


Figure 25. Differential expression levels of miR-486-5p between monolayer and CSC derived from T-84, HT-29, HCT-116 cell lines as measured by Real-time RT-PCR

8.3.7. Effect of miR-486-5p mimic and inhibitor on expression of stemness genes

The expression of five stemness factors: *SOX2*, *NANOG*, *OCT4*, *KLF4*, and *c-MYC*, was evaluated in HCT-116 cell line, both CSCs and monolayer cells, under the effect of either the miR-486-5p mimic or inhibitor and normalised to the respective controls. The HCT-116 cell line was chosen because, it has been shown as a better model to induce the CSC phenotype in comparison to HT-29, which has a greater tendency to differentiate faster than HCT-116 (Yao et al., 2005), whereas T-84 cell line is a CRC in vitro model derived from lung metastasis. In monolayer of HCT-116, the expression levels of OCT4 and KLF4 were higher in the controls than in the mimic-treated cells, whereas the expression of all the genes was higher in the inhibitor-treated cells than in either the controls or the mimic-treated cells with the only exception of SOX2, slightly more expressed in the mimic-treated cells, with no statistical significance. The OCT4, KLF4, and cMYC expression levels were significantly different between cells in all treatment comparisons

(Figure 26A and Table 4). In the HCT-116 CSCs, all the stem factors were highly expressed in all the controls than in the mimic-treated cells, except for SOX2, whereas the expression of all the genes was higher in the inhibitor-treated cells than in the control and mimic-treated cells. The differences in all the comparisons were statistically significant, except for the difference in OCT4 among the control and mimic-treated cells (Figure 26B and Table 5). As expected, comparing the effect of control mimic on stem cell genes in monolayers and CSCs, it shows overexpression of stem cell genes in CSCs (Figure 26C). The same effect was evaluated for the counter inhibitor with an upregulation observed for most of the factors except for Nanog and Oct4 (Figure 26D).

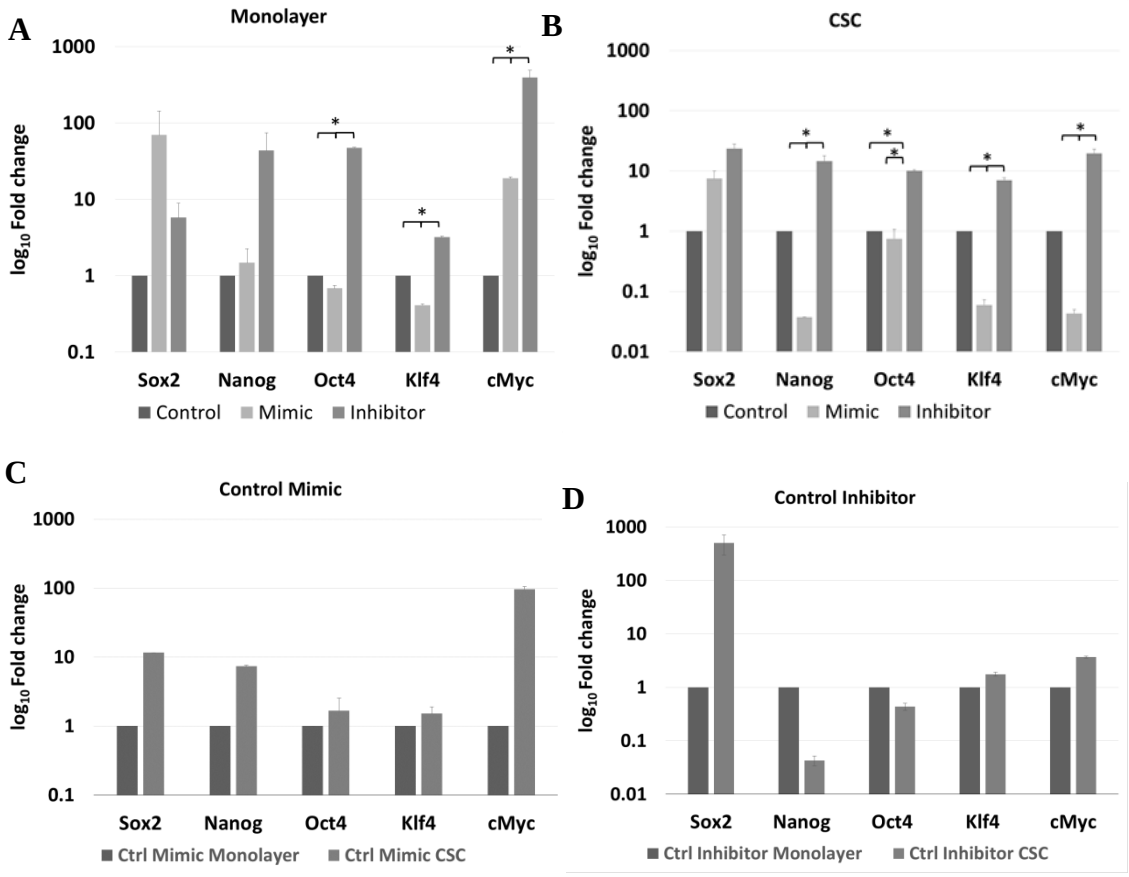


*Figure 26.. Real-time RT-PCR quantification of stemness factors (Sox2, Nanog, Oct4, Klf4 and cMyc) in monolayer (**A**) and in CSCs (**B**) after treatment with miR-486-5p mimic or inhibitor. Each treatment was normalized to the respective control (mimic vs. control mimic and inhibitor vs. control inhibitor) represented in this figure as unique control (both controls were indicated as reference and imposed equal to 1). (**C**) real-time RT-PCR quantification and comparison of the differential expression of stemness factors (Sox2, Nanog, Oct4, Klf4 and cMyc) in monolayer and CSCs after control mimic treatment; (**D**) real-time RT-PCR quantification and comparison of the differential expression of stemness factors (Sox2, Nanog, Oct4, Klf4 and cMyc) in monolayer and CSC after control inhibitor treatment. The symbol * indicates differences supported by statistical significance.*

Table 4. p values referred to fig. 26 for the cells cultured in monolayer.

Monolayer			
Stem factor	Control vs mimic	Control vs inhibitor	Mimic vs inhibitor
SOX2	0,18	0,06	0,21
NANOG	0,41	0,07684	0,08
OCT4	<0.01	<0.01	<0.01
KLF4	<0.01	<0.01	<0.01
MYC	<0.01	<0.01	<0.01

Table 5. p values referred to fig. 26 for CSCs.

CSCs			
Stem factor	Control vs mimic	Control vs inhibitor	Mimic vs inhibitor
SOX2	0,01	<0.01	<0.01
NANOG	0,01	<0.01	<0.01
OCT4	0,81	<0.01	<0.01
KLF4	0,22	<0.01	<0.01
MYC	<0.01	<0.01	<0.01

8.3.7.1. Microarray genomic analysis of the miR-486-5p effect

From our results, we conceived a working hypothesis: miR-486-5p exerts a negative regulatory effect on the CSC phenotype in CRC through the activation of genes that inhibit the CSC phenotype and through the down-regulation of genes that promote the CSC phenotype. To test the working hypothesis (depicted in Figure 27), mRNA microarrays were used, to analyse the effects of the induced up or down-regulation of miR-486-5p on HCT-116 cells line, in monolayer and CSCs. This approach allowed us to eventually individuate the effect of miR-486-5p on the genes involved in CSCs phenotype, being progressively promoted or inhibited in the passage from monolayer to CSC and viceversa. 135,750 transcripts were tested, and 2575 of these showed expression patterns consistent with our working hypothesis (empirical $p < 0.05$ after permutation-based correction for multiple testing). Genes consistent with our hypothesis were then filtered to a list of 240 genes with a role in the CSC phenotype. This list was produced considering the four principal biological pathways involved in CSCs: Wnt,

Notch, Hedgehog, and TGF-β. Several of 16 genes were consistent with our working hypothesis. The weighted Kolmogorov–Smirnov enrichment test indicated that genes adherent to our working hypothesis were enriched in the four biological pathways previously described (empirical $p < 0.01$). These results indicate that miR-486-5p regulates the expression of important genes involved in CSC-related pathways. We observed the promotion of expression by miR-486-5p for 11 genes (*PLK1*, *FRZB*, *TCF7L2*, *SMAD4*, *NFATC1*, *KDM4C*, *PPARD*, *KLF4*, *CDX2*, *STAT3*, and *EP300*), up-regulated when miR-486-5p was overexpressed and down-regulated when it was inhibited. On the other hand, we observed that miR-486-5p inhibits the expression of 5 genes (*NRCAM*, *JUN*, *WNT10A*, *FZD8*, and *NODAL*) down-regulated when miR-486-5p was overexpressed and up-regulated when it was inhibited. All these genes showed the same behavior in monolayer cells and CSCs, when analysed independently. Furthermore, 8 of these genes showed progressive up or down-regulation in the transition from monolayer cells to CSCs with ptrend < 0.05: *PLK1* (ptrend = 0.0001), *FRZB* (ptrend = 0.0002), *TCF7L2* (ptrend = 0.0004), *SMAD4* (ptrend = 0.016), *NFATC1* (ptrend = 0.02), *KDM4C* (ptrend = 0.04), *NRCAM* (ptrend = 0.008), and *JUN* (ptrend = 0.04). The other eight genes showed no progressive up- or down-regulation in the transition from the monolayer to the CSC phenotype, then the p values were calculated for each condition: *PPARD* (monolayer ptrend = 0.09; CSCs ptrend = 0.01), *KLF4* (monolayer ptrend = 0.09; CSCs ptrend = 0.03), *CDX2* (monolayer ptrend = 0.02; CSCs ptrend = 0.06), *STAT3* (monolayer ptrend = 0.08; CSC ptrend = 0.01), *EP300* (monolayer ptrend = 0.09; CSCs ptrend = 0.03), *WNT10A* (monolayer ptrend = 0.06; CSCs ptrend = 0.02), *FZD8* (monolayer ptrend = 0.09; CSCs ptrend = 0.05), and *NODAL* (monolayer ptrend = 0.01; CSCs ptrend = 0.03) (Figure 28).

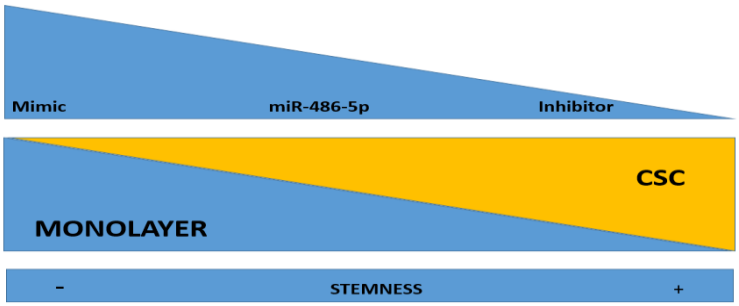


Figure 187. Working hypothesis: miR-486-5p may play an inhibitory effect on the stemness of CSCs. When it is up-regulated by mimic, there is an induction of stemness loss. When the miRNA is down-regulated (by an inhibitor), it induces stemness in the recipient cells. miR-486-5p inhibition drives to CSC phenotype, while its promotion inhibits the CSC phenotype.

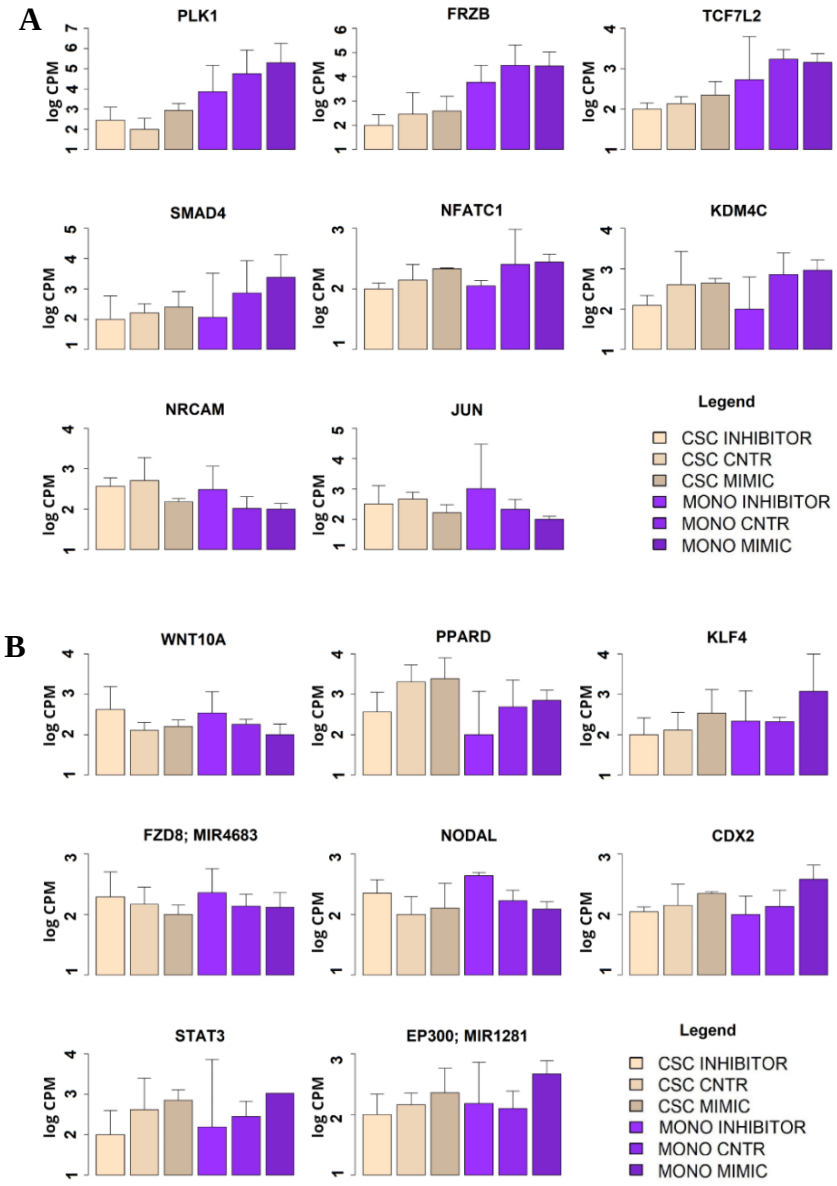


*Figure 28. Expression levels of the genes involved in the four main regulatory pathways of the CSC phenotype (Wnt, Hedgehog, Notch and TGFβ) as measured by microarray. Expression levels were measured in two models of HCT-116: CSC and monolayer after treatment with has-miR-486-5p mimic or inhibitor. (**A**) Statistically significant genes with a progressive increment or decrement in the passage from monolayer to CSCs; (**B**) statistically significant genes with the same behavior between monolayer and CSCs.*

9. DISCUSSION

The main focus of the present thesis was to identify miRNAs involved in CRC and in the CSC phenotype, to better understand the onset and development of this disease. The improvement of knowledge on this issue could offer further useful tools for the fight against CRC, improving diagnosis, prognosis and treatment. In order to achieve this result, the role of some miRNAs in the onset and development of CRC has been studied through the analysis of human samples and cell models, with particular attention to their effect on the level of the CSC phenotype. To do this two approaches were used, on the one hand, a careful analysis of the literature and selected a set of miRNA known to play a role in CRC and its metastatic development; on the other hand, independently from literature data, a miRNA signature analysis that resulted in the selection of a miRNA with a protective behavior against the CRC and CSC phenotype. To this end, the analysis of miRNoma in a group of subjects and subsequent validation and functionality *in vitro* studies were performed.

We examined putative colorectal CSC-associated miRNAs both *in vitro* and in CRC patients, in three different CSC models obtained from human established CRC cell lines and cancer tissue, healthy tissue and serum of adult CRC patients, respectively. Then, we correlated the miRNA levels with clinical data.

As mentioned before for the first approach, a set of miRNAs was used both in the cell line model as in tissues derived from CRC patients. For the in vitro tests were used: a) HCT-116, HT- 29 and T-84 colorectal CSC models; b) HCT-116, HT-29 and T-84 monolayer-cultured cell lines; and c) the normal colonic cell line CCD-18Co allowing to distinguish the miRNAs with a role in normal, cancerous and CSC in vitro models. These miRNAs were highly deregulated in the cancer tissue and serum of CRC patients, suggesting new options for the development of miRNA-directed clinical tools. First of all, miR-21 is known to target the transcript of the oncosuppressor gene *PTEN*, resulting in EMT promotion, cell migration and the invasion of CRC organoids by inhibiting TIAM1

(Cottonham et al., 2010). Here, miR-21, together with miR-221 were selected, as positive controls for the miRNA assays because of their function to the self-renewal control in CSC and the reduction of AKT phosphorylation in CRC (Roy et al., 2013). The data here presented show an upregulation of miR-21 in CSCs and in the cancerous samples. An exception was present in the HCT-116 CSC, a result that looks in contrast with the literature that describes the upregulation of miR-21 in the CSC derived from HCT-116 cells. Otherwise, this different result may be attributable to a different stem cells-enrichment methods and markers (P. Xu et al., 2018; Y. Yu et al., 2012, 2013, 2015). Furthermore, it was shown an upregulation of miR-221 in HCT-116 and T-84 CSCs, as well as in cancer tissue and serum of CRC patients when compared with the healthy counterpart. This result is in accordance with the literature, which reports the role of miR-221 in cancer cell proliferation by inhibiting CDKI, which promotes G0/G1-to-S cell-cycle phase advancement (K. Sun et al., 2011). In contrast, in HT-29 cell line, miR-221 was downregulated in the CSC model, although this data is consistent with another study that showed the downregulation of miR-221 in CD133+ HT-29 CSC relative to its expression in differentiated CD133- HT-29 cells (H. Zhang et al., 2011). This evidence may suggest that HT-29 cells could adopt other CDKI regulatory mechanisms in cell-cycle progression. An unexpecting data was the higher expression of miR-221 in CCD-18Co cells in respect to HCT-116 or T-84 cells, suggesting that miR-221 also plays a role in the replication of normal colon cells. Furthermore, the upregulation of miR-221 was observed in serum from CRC patients, and more significantly in the LOW and no-Met patients, suggesting that miR-221 acts early in CRC. With a positive correlation with age, suggesting a possible CRC initiation biomarker role, especially in younger patients. Therefore, miR-221 may offer a tool for the blood-based early screening of CRC. Also, if considered the low number of the population examined, further studies are needed.

miR-18a belongs to the 17-92 cluster, the less known for its oncogenic role in CRC

(Tsuchida et al., 2011), but also its upregulation has been described in several tumours (Z. Luo et al., 2013; L. Zhou et al., 2018), including CRC (Tsuchida et al., 2011). Other authors have described that miR18a acts as a balancing miRNA of the 17–92 cluster, acting as an anti-oncomiR (miRNA with oncosuppressor activity) by inhibiting CDC42 (Humphreys et al., 2014). It may also targets the transcripts of the KRAS oncogene, which is involved in the initiation and progression of CRC (Tsang & Kwok, 2009). We showed an upregulation of miR-18a in all cancer models, both in vitro and in patients. In cell line, comparing CSC models with the respective monolayer, we observed a downregulation of miR-18a in the CSC of HCT-116 and HT-29, result subverted in the T-84 model and when an average is made between the values of all the CSC models, compared to the average valuses of monolayers. In patients, miR-18a was dramatically upregulated, in particular in the serum of metastatic patients. The Spearman's correlation analysis, showed a positive correlation between miR-18a and metastasis and a negative one with age, suggesting that high level of miR-18a in the blood of adult patients, rather than in younger patients, could be indicative of the presence of CRC metastasis. Thereby supporting a potential role for miR-18a as a serum-based cancer biomarker (Komatsu et al., 2014), especially valid for adult CRC metastatic patients. However, due to the contradictory results between in vitro and patients, appropriate mechanistic studies are required to clarify the real function of this miR in the stemness properties of cells and in cancer progression and metastasis.

miR-210 is one of the most important miRNA involved in hypoxia (Kulshreshtha et al., 2007). Furthermore, it is known to induce apoptosis in CRC cells, including HCT-116 cell line (Tagscherer et al., 2016), and it is also involved in cell migration and invasion, promoting CRC metastasis (A. Qu et al., 2014). In our data, with the exception for HCT-116, miR-210 was upregulated in CSCs relative to its expression in the corresponding monolayer cell cultures and in cancer tissue in comparison to its expression in healthy

tissue. These results are in accordance to other studies that reported the upregulation of miR-210 in adenocarcinomas relative to its expression to the controls, and correlation with CRC TNM and clinical stage (Sabry et al., 2019). In line with Tagscherer et al. (2009), we observed the downregulation of miR-210 in HCT-116 CSC. The authors purposed that miR-210 downregulation in HCT-116 CSC, could represent an apoptosis-avoiding molecular mechanism, in consideration of the apoptosis-inducing role of miR-210 in these cells (Tagscherer et al., 2016).

In a number of cancers, miR-31 is classified as an oncosuppressor, even when defects in the TP53 pathway occurs. However, in CRC it is linked to disease progression, particularly in the non-conventional pathway of serrated colorectal lesions, which requires the mutation or deregulation of BRAF, but not of APC (Nosho et al., 2014). Here, it is shown an upregulation of miR-31 in cancer cells relative to the healthy cells, and in CSCs relative to monolayers, with the only exception being in HCT-116 CSC. These trends are consistent with studies demonstrating roles for miR-31 loss of function in chemoresistance, cell migration and invasion, but not in the proliferation of HCT-116 cells (C. J. Wang et al., 2010). This differs from its role in BRAF- and TP53-mutated HT-29 cells, in which its function is closely associated with cell proliferation (R. S. Xu et al., 2013). In contrast to our data for HT-29 CSC, a slight miR-31 downregulation in CD133+ HT-29 cells with stem-like properties were reported by Zhang et al. (H. Zhang et al., 2011); however, the usefulness of CD133 as a unique marker of colorectal CSC is debated for several cancer cell types (Dittfeld et al., 2009; J. Y. Zhou et al., 2016). In our data, miR-31 was upregulated in both cancer tissue and serum when compared to healthy tissue. Moreover, the highest expression was observed in the HIGH and metastatic patients, suggesting that miR-31 is a candidate CRC-specific diagnostic and prognostic marker.

In addition, miR-34a was downregulated in cancer models, which is in line with its role in downregulation of two of the main pathways of CSC of CRC, WNT(Cai et al., 2018)

and NOTCH1 (Bu et al., 2013)(Lihua Wang et al., 2016). Nevertheless, our data showed unexpectedly lower miR-34a expression in the HCT-116 and HT-29 cell lines relative to its expression in HCT-116 and HT-29 CSCs, suggesting possible WNT pathway activity also in the monolayer cell cultures. However, in the T-84 CSCs, the expression of miR-34a was higher compared to the relative monolayer, as expected. In our patients, no differences in miR-34a expression were detected between cancer tissue and healthy tissue, but there was only an increasing trend in cancer tissue, while miR- 34a levels were significantly lower in serum than in healthy tissue.

miR10b is a less studied miRNA, otherwise, some authors reported that its loss induces WNT signalling (Stadthagen et al., 2013) and that stem-like cells in head-and-neck cancer recruit miR-10b to promote chemoresistance, cell migration and invasion (Bourguignon et al., 2016). In our in vitro CSC models, miR-10b was slightly deregulated in relation to its expression in monolayer cells, with its highest expression in T-84 CSC model. However, in this study miR-10b was also the most significantly downregulated miRNA in cancer tissue and serum in the LOW group and in both metastatic and non metastatic patients, suggesting that its early downregulation is associated with CRC initiation and progression.

In the case of miR-16 they are controversial studies, since some works suggest it as oncosuppressor, downregulated in CRC (Qian et al., 2013) and able to inhibit cell proliferation by modulating the TP53/survivin pathway (Q. Ma et al., 2013). Moreover, its expression may activate apoptosis in CRC (Sam et al., 2016). Otherwise, some studies highlighted that high levels of miR-16 are present in CRC tissues (Hasáková et al., 2017) and/or its association with overall survival (Diamantopoulos et al., 2017)(G. Xiao et al., 2014). In our study, a dramatical downregulation of miR-16 was observed in all the CSC models, in accordance with the literature. In contrast, miR-16 was upregulated in cancer tissue and serum in respect to healthy tissue, particularly in the LOW grade and non

metastatic patients, suggesting that high level of miR-16 in cancer tissues could be an early diagnostic marker of CRC. Because this miRNA was downregulated in all CSC types, its reduced expression in patients with CRC could be a marker of poor prognosis.

We have also noted several similarities in the miRNA expression patterns in CSCs and healthy colonic fibroblast cells. This last unexpected result suggests that fibroblasts, mesenchymal cells and CSCs share some structural and molecular traits (Fender et al., 2015; Ju et al., 2014; Xiaochao Zhang et al., 2018).

In parallel, in order to do a biomolecular characterization of CSCs in CRC, we also used a broader approach, starting from the analyses of the miRNome of 47 subjects, divided into three groups: healthy controls, CRC non metastatic patients, CRC metastatic patient. This preliminary analysis guided us to the identification of a candidate miRNA, with a protective role against CRC metastasis, miR-486-5p, on which a meta-analysis has been conducted in serum and tissues, involving a large number of patients. Then, to understand its role in CSC, in vitro functional and genetic characterization was performed. Most in detail, the preliminary miRNome analysis from the pooled RNA per group showed that miR-486-5p was progressively down-regulated in sera as the CRC pathology progressed, with the lowest expression levels in the metastatic group. In order to have a most detailed understanding about behavior of miR-486-5p, it was quantified in each individual patient by real-time RT–PCR. From this quantification, two main results emerged: first, the real-time RT-PCR analysis overturned the difference between the controls and the non-metastatic tumor group observed when miRNAs were profiled by small RNA-sequencing. The two techniques, in part, offered contrasting results, and this is probably due to the different analysis approach used: contrary to real-time RT-PCR, in miR–seq the samples were merged in pool per group, and the presence of outlier patients may have induced bias. Furthermore, this last data was not supported by statistical significance. More specifically, miR-486-5p was down-regulated in the control group in relation to the non

metastatic tumor group (stage I–II). When the CRC groups were merged in a single group (all stages) and compared with the control group, miR-486-5p was up-regulated in the tumor group, data strongly supported by a meta-analysis conducted on thousands of patients. The diagnostic power of miR-486-5p was tested by performing a ROC miR-486-5p in the sera of our patients, in a model which includes other predictor factors such as age, sex, smoking history, levels of CEA, and levels of alkaline phosphatase. Further, the prognostic utility of miR-486-5p was tested in sera of our CRC patients via a Cox regression model to evaluate 5-years survival after surgery. The model included age, sex, tumor stage and grade, CEA, and alkaline phosphatase as covariates. Our results show that the inclusion of miR-486-5p in a prediction model increased both prognostic and diagnostic power (although without strong statistical significance, likely as a consequence of the lack of statistical power). Our results, although if conducted on small sample size, and having no strong statistical support, deserves further investigation to understand whether miR-486-5p can become a useful diagnostic and prognostic biomarker, possibly in combination with those currently used in clinical practice. To clarify the role of miR-486-5p in CRC, we compared its levels in stool from cases and controls, to establish its usefulness in CRC screening (Grossi, 2019; Smith et al., 2019), and in the intestinal mucosa tissues affected by the disease. Due to the difficulty of finding datasets with information on the miRNA expression in stool, this matrix was analysed with small RNA-sequencing in another cohort of CRC patients and controls. For mir-486-5p, the results were in agreement with those of the serum, with an up-regulation of miR-486-5p in CRC patients was observed. Interestingly, in tissue samples, the expression levels of miR-486-5p were in the opposite direction compared to those of serum and stool. Another data worthy of attention emerged from the comparison between the results of the real-time RT-PCR and the small RNA sequencing, is their accordance when metastatic CRC patients were compared with non metastatic CRC patients, confirming lower expression of miR-

486-5p in the sera of metastatic patients. miR-486-5p is a scarcely investigated miRNA in the literature and these preliminary results incentive us to further investigate its utility as prognostic factor. In consideration of the important role of the CSC in metastasis of CRC and other tumors (Reya et al., 2001; Singh & Settleman, 2010), we investigate the role of miR-486-5p in a model representative of this population. To this end CSC models were established from three CRC cell lines HCT-116, HT-29, and T-84. In this model a downregulation of miR-486-5p was observed in comparison to the monolayer counterpart (representative of non-metastatic tumor), supporting our hypothesis of an involvement of miR-486-5p in metastasis by influencing CSC phenotype. The understanding about miR-486-5p in CSCs was further investigated studying it in HCT-116 cell line, in which the up and down-regulation of miR-486-5p were induced in both cellular models, allowing to confirm the negative function of miR-486-5p against the CSC phenotype. The effects of miR-486-5p on stemness factors *SOX2*, *OCT4*, *KLF4*, *cMYC* and Nanog were then examined by real-time RT–PCR, and the effects on the four principal pathways of CSCs (Wnt, Hedgehog, Notch, and TGF-β) were analysed by microarray analysis.

Several papers describe the up-regulation of miR-486-5p in the blood of cancer patients, including lung cancer (Świtlik et al., 2019) and CRC (X. Liu et al., 2018; S. Yan et al., 2017). We observed this behavior in a group of individuals and validated it with an extensive meta-analysis. Furthermore, our preliminary data were also confirmed in stool samples. The presence of occult blood in faecal samples is routinely used in CRC screening (Grossi, 2019), and correlations between the concentrations of other miRNAs in blood and stool have been demonstrated, as for miR-486-5p (Wu et al., 2017). On the other hand, in tissues the expression of miR-486-5p was higher in healthy mucosae than in tumor. The lower expression of miR-486-5p in CRC tissues has been previously reported in the literature, where this miRNA was described as an oncosuppressor (X. Liu et al., 2018; X. Yan et al., 2019; Xiaoguang Zhang et al., 2016). The contrasting behavior

of miR-486-5p between blood and tissues has been recently discussed by Liu et al. (X. Liu et al., 2018). Many other authors have investigated the opposite behaviors of certain miRNA expression levels in tissues and serum (Pardini & Calin, 2019; Pigati et al., 2010). In some cases, tissue/serum opposite miRNA expression is due to the endocrine role played by some miRNAs, that once released from the primary tumor into the bloodstream by exosomes, are able to condition other cells, or the tumor microenvironment (Pardini et al., 2019; Pardini & Calin, 2019). Alternatively, some miRNAs are released from cells without the exosomal use and are carried by proteins, such as AGO (Kamal & Shahidan, 2020; Turchinovich et al., 2011) and apolipoproteins (Niculescu et al., 2015), via active or passive means, such as leakage from cells after injury, chronic inflammation, apoptosis, or necrosis (Kamal & Shahidan, 2020; Redis et al., 2012). This mechanism is considered to account for 90% of circulating miRNAs (Arroyo et al., 2011). Turchinovic et al. assumed that the greatest amounts of extracellular miRNAs are conjugated to AGO proteins raider then included in exosomes, and may be released into the bloodstream after cell death. Furthermore, the authors supposed that released miRNA may plays a role in this phenomenon (Turchinovich et al., 2011). These observations may clarify why miR-486-5p is down-regulated in the sera of CRC metastatic patients. We hypothesized a link between the down-regulation of miR-486-5p in serum and its absence in CSCs, as observed in our study. It is conceivable that the high serum levels of miR-486-5p in the early stages of CRC patients could be associated with its release from death cells (Turchinovich et al., 2011), which may be induced by the miRNA itself during the performance of its tumor-suppressive function (X. Yan et al., 2019)(Xiaoguang Zhang et al., 2016), among which apoptosis (M. Zhang et al., 2020). On the other hand, the absence of miR-486-5p can drive to a worsening of cell fate, a condition that may have a potential role in CSC development and metastasis insurgence, although more specific and in-depth studies are necessary to support this hypothesis.

The role of miR-486-5p in the CSC phenotype and in metastasis has been described in tissues for different tumors (X. Yan et al., 2019; Xiaoguang Zhang et al., 2016), although in glioblastoma, has been described the opposite behavior (Lopez-Bertoni et al., 2020). This interesting result invited us to further investigate the role of miR-486-5p in CSC of CRC, by inducing forced expression or the inhibition of this miRNA in cell lines, and to study its effect to the genes involved in CSC phenotype. The treatment with mimics or inhibitors for miR-486-5p in HCT-116, both in monolayer and CSCs, allowed us to show its negative effect on the stemness factors *SOX2*, *OCT4*, *cMYC*, *KLF4* (Takahashi & Yamanaka, 2006), and NANOG (Pan & Thomson, 2007). The most evident negative effect of miR-486-5p was observed for *KLF4* and *OCT4* in both monolayer cells and CSCs, followed by *cMYC* and *NANOG*, with the greatest effect in CSCs; however, no clear effect on *SOX2* expression was observed. *KLF4* is involved in a series of CSC processes, including cell differentiation, the maintenance of pluripotency, self-renewal, and apoptosis (Hadjimichael et al., 2015; Leng et al., 2013), and it is overexpressed in CSCs of CRC (Leng et al., 2013). *KLF4* promotes a series mechanisms with a key role in metastasis, such as EMT, migration, invasion (Hadjimichael et al., 2015) and TP53 inhibition, with as a consequence the *NANOG* promotion (Takahashi & Yamanaka, 2006; W. Zhang et al., 2006). We also showed the negative effect of miR-486-5p on OCT4, an effect already observed in liver cancer (X. Yan et al., 2019), highlighting the miR-486-5p involvement in the inhibition of CSC phenotype. In fact, OCT4 is overexpressed in cancer (R.-J. Kim & Nam, 2011), including CRC, with involvement in malignancy and metastasis (Amini et al., 2014). Moreover, *OCT4* in combination with *SOX2* regulates *NANOG* in the stemness process (Hadjimichael et al., 2015; Pan & Thomson, 2007; Takahashi & Yamanaka, 2006). *NANOG* is a dispensable factor in the induction of stemness and is also important in its maintenance (Takahashi & Yamanaka, 2006), highlighting a late activation of *NANOG* in the stemness process in accordance with our

results, in which the effects of miR-486-5p on NANOG were more evident in CSCs. The role of *NANOG* in CSCs and CRC has been jet demonstrated (Ibrahim et al., 2012). We observed the most important effect of miR-486-5p on c-*MYC* in CSC, such as for *NANOG*. c-*MYC* is one of the most important factors in cell biology; in fact, it regulates nearly 15% of all human genes (Klapproth & Wirth, 2010) and it is involved in the biology of CSCs (B. S. Xu et al., 2020). The effect of miR-486-5p on c-*MYC* has been previously observed in CRC (Su et al., 2018). Furthermore, by using miRpath we find that c-*MYC* is a direct target of miR-486-5p (Vlachos et al., 2012). As mentioned before, we did not detect a clear effect of miR-486-5p on *SOX2* expression. It is a transcription factor involved in embryonal development, pluripotency maintenance, and cell self-renewal (Takahashi & Yamanaka, 2006), with roles in tumors (Meng et al., 2010; Nagata et al., 2014), such as CRC (Amini et al., 2014; Talebi et al., 2015), and in the development of CSCs (Hadjimichael et al., 2015). The relationship between *SOX2* and miR-486-5p is controversial and, although *SOX2* expression is promoted by miR-486-5p in some tumors, such as glioblastoma (Lopez-Bertoni et al., 2020), it inhibits *SOX2* in liver cancer (X. Yan et al., 2019). This corroborates our observation that there is no link between miR-486-5p and SOX2 expression.

The CSCs phenotype is regulated by four principal pathways: Wnt, Notch, Hedgehog, and TGF-β (Talebi et al., 2015). Our microarray analyses data highlighted the involvement of miR-486-5p in CRC biology and in the molecular mechanisms of CSCs by regulating the above-mentioned pathways. miR-486-5p promotes the expression of five genes with oncosuppressive role and thereby with a negative effect on the CSC phenotype: FZB (Deshmukh et al., 2019; Y. Qu et al., 2008), *SMAD4* (Miyaki et al., 1999; Zhao et al., 2018) *NFATC1* (Horsley et al., 2008; Pudova et al., 2018), *CDX2* (Sakamoto et al., 2017; Suh & Traber, 1996) and *EP300* (Chan & La Thangue, 2001; Krubasik et al., 2006) (Figure 29). On the other hand, it inhibits the expression of further five genes with oncogenic and CSC-promoting

roles: *NRCAM* (Conacci-Sorrell et al., 2002; J. C. Yu et al., 2011), *JUN* (Dong et al., 2019; Vogt, 2002), *WNT10A* (J. Li et al., 2019; Long et al., 2015), *FZD8* (S. Sun et al., 2014; L. Xu et al., 2016), and *NODAL* (Lonardo et al., 2011; L. Xiao et al., 2006) (Figure 29). Moreover, miR-486-5p showed a positive effect on the expression of *PLK1*, involved with a proper cell division process (Barr et al., 2004) and with controversial behavior in cancer, in which it is described both as an oncogene (Donizy et al., 2016) and an oncosuppressor (Strebhardt et al., 2018). Finally, miR-486-5p also promotes the expression of five genes with promoting cancer roles: *KDM4C* (Gregory & Cheung, 2014; Yamamoto et al., 2013), *PPARD* (Y. Liu et al., 2019; Zuo et al., 2017), *KLF4* (Leng et al., 2013), *STAT3* (Takahashi & Yamanaka, 2006; X. Yan et al., 2019), and *TCF7l2* (Shao et al., 2003; Tkach et al., 2013). The up-regulation of *STAT3* and *TCF7l2* in the presence of this miRNA may be a consequence of the miR-486-5p-mediated activation of *EP300* (Sakamoto et al., 2017) and *FZB* (Liang et al., 2019). (Figure 29).

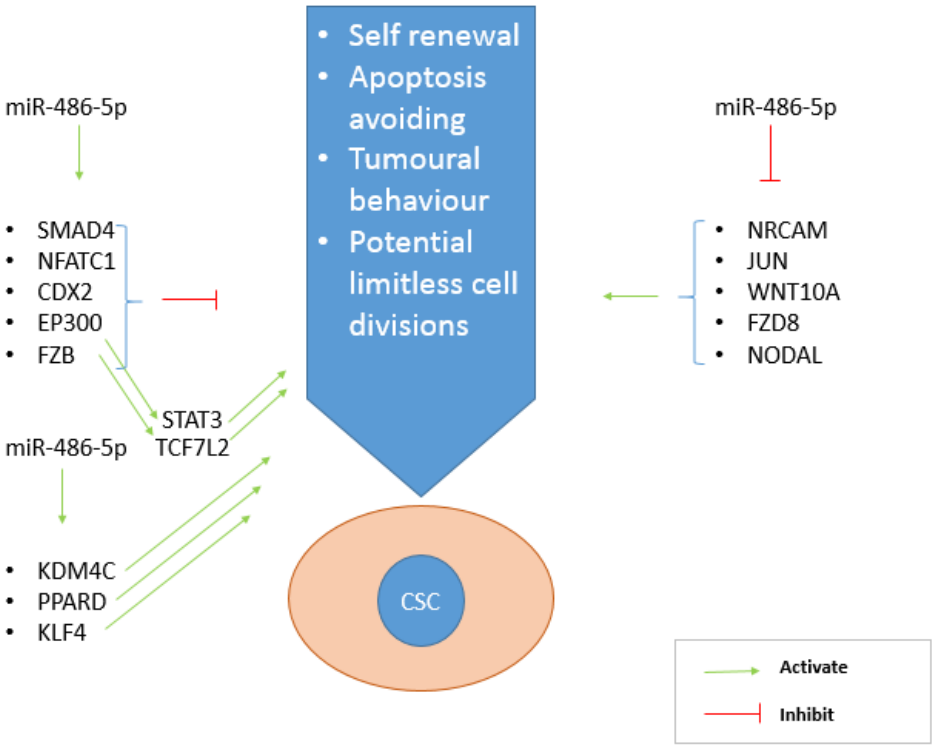


Figure 29. Main genes regulated by miR-486-5p thanks to its inhibitory role on CSC phenotype. On the left, the genes activated by the miRNA, on the right those inhibited. miR-486-5p promotes the effect of CSC inhibiting genes (SMAD4, NFATC1, CDX2, FZB and EP300), but also inhibits the action of CSC promoting genes (NRCAM, JUN, WNT10A, FZD8, NODAL).

10. CONCLUSIONES

1. Se observó una asociación del miR-18a con el desarrollo de tumor y de metástasis y con el fenotipo de CMC.

2. Se demostró una relación entre el fenotipo de CMCs y los miARNs miR-21, miR-210, miR-34a y miR-16, observándose una sobreexpresión para los tres primeros y una regulación descendente en el último.

3. Se observó una asociación entre el tumor y el miR-31b, tanto en los tejidos como en los cultivos celulares; y, además, destacó una sobreexpresión de dicho miARN en el suero de los pacientes metastásicos.

4. Una asociación con regulación descendente se observó entre el miR-10b y el CCR, tanto en los tejidos tumorales como en el suero de los pacientes no metastásicos.

5. Nuestros resultados sobre la relación de los miARNs miR-21, miR-210, miR-10b, miR-31b y miR-18a con las CMCs de CCR indican que son necesarios más estudios para determinar su papel como marcador predictivo y de pronóstico en el CCR.

6. El análisis del miRNoma de un grupo de pacientes con CCR, junto con el meta-análisis en una gran población de individuos, permitió destacar el valor diagnóstico y pronóstico precoz del miR-486-5p.

7. El miR-486-5p mostró un comportamiento diferente al comparar los resultados en los tejidos y en el suero de los pacientes con CCR, destacando una desregulación en los tejidos y una sobreexpresión en el suero.

8. Nuestros resultados indican un vínculo entre la expresión del miR-486-5p en el suero y en las heces de los pacientes con CCR. Además, también demuestran el papel inhibidor de los miARNs hacia el fenotipo de CMC en CCR.

9. El miR-486-5p mostró ser una potencial herramienta en el diagnóstico y pronóstico del CCR en asociación con otros marcadores tumorales utilizados actualmente en los servicios oncológicos.

11. CONCLUSIONS

1. An association primarly with the tumor, than with the metastasis and CSC phenotype was observed for miR-18a.

2. An association between miRNAs and CSC phenotype was demonstrated in miR-21, miR-210 miR-34a and miR-16, observing an overexpression for the first three and a downregulation of the last one.

3. An association between tumor and miR-31b has been observed in both tissues and cell models, furthermore it was highlighted an ovrexpression in serum of metastatic patients.

4. The association between miRNAs downregulation and CRC was observed for miR-10b, and in both tumor tissues and serum of non-metastatic patients.

5. Our results about miR-21, miR-210, miR-10b, miR-31b and miR-18a related to CRC CSCs indicate that further studies are necessary to determine their role as miRNA singnature with prognostic and predictive potential in CRC.

6. The analysis of the miRNoma of a group of CRC patients together the meta-analysis in a large population of individuals allowed to highlight the early diagnostic and prognostic value of miR-486-5p.

7. miR-486-5p showed different behavior between tissues and serum of patients, highlighting its downregulation in tumoral tissues and its overexpression in serum of CRC patients.

8. Our results indicate the link between the expression of miR-486-5p in serum and faeces of CRC patients and the inhibitory role of miRNAs towards the CSC phenotype in CRC.

9. miR-486-5p showed as a powerful potential tool in the diagnosis and prognosis of CRC in association with other tumor markers currently used in oncology services.

12. CONCLUSIONI

1. Si osservata un'associazione del miR-18a, dapprima col tumore e poi con la metastasi e con il fenotipo CSC.

2. Viene dimostrata un'associazione tra i miRNA e il fenotipo CSC per miR-21, miR-210, miR-34a e miR-16, osservando un'iperespressione per i primi tre e la downregolazione per l'ultimo.

3. Si osserva un'associazione tra tumore e il miR-31b, sia nei tessuti che nei modelli cellulari, inoltre si osserva una sua sovraespressione nel siero dei pazienti metastatici.

4. Un'associazione tra l'ipoespressione dei miRNA e il CCR viene osservata per il miR-10b, questa viene inoltre registrata sia nei tessuti tumorali e nel siero dei pazienti non metastatici.

5. I nostril risultati su miR-21, miR-210, miR-10b, miR-31b and miR-18a relativi al CCR e CSCs, indicano la necessità di ulteriori studi, al fine di comprendere meglio il loro ruolo in questo fenomento e al fine di valutarne la potenzialità come marcatori diagnostici e prognostici.

6. L'analisi del miRNoma di un gruppo di pazienti CCR, unitamente alla meta-analisi di una larga popolazione di individui, ha consentito di evidenziare il potere diagnostico precoce e prognostico del miR-486-5p.

7. Il miR-486-5p mostra un comportamento differente tra mucosa e siero dei pazienti, evidenziando la sua ipoespressione nei tessuti tumorali e la sua sovraespressione nel siero dei pazienti CCR.

8. I nostril risultati indicano un legame tra l'espressione del miR-486-5p nel siero dei pazienti CCR metastatici e il suo ruolo inibitorio verso il fenotipo CSC.

9. Il miR-486-5p si dimostra un potenziale strumento nella diagnosi e nella prognosi del CCR, in associazione con altri marcatori attualmente impiegati a tale scopo.

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14. ANNEXES

14.1. CURRICULUM

ACADEMIC ACHIEVEMENTS:

-Bachelor in Biology
University of Sassari, Iraly, 2010

-Experimental and Applied Biology
University of Sassari, Italy 2013

-Italian qualification to the profession of biologist
University of Sassari, Italy, 2013

PUBLICATIONS:

Forte, G, Bocca, B, **Pisano, A**, Collu, C, Farace, C, Sabalic, A, Senofonte, M, Fois, A, G, Mazzarello, V, L, Pirina, P, Madeddu, R.
The levels of trace elements in sputum as biomarkers for idiopathic pulmonary fibrosis
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DOCUMENT TYPE: Article
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Quartile: Q1; Impact Factor (JCR): 5.77

Farace, C., Fenu, G., Lintas, S., Oggiano, R., **Pisano**, **A**., Sabalic, A., Solinas, G., Bocca, B., Forte, G., Madeddu, R.
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Pisano, A., Griñan-Lison, C., Farace, C., Fiorito, G., Fenu, G., Jiménez, G., Scognamillo, F., Peña-Martin, J., Naccarati, A., Pröll, J., Atzmüller, S., Pardini, B., Attene, F., Ibba, G., Solinas, M.G., Bernhard, D., Marchal, J.A., Madeddu, R.
The inhibitory role of mir-486-5p on csc phenotype has diagnostic and prognostic potential in colorectal cancer
(2020) Cancers, 12 (11), art. no. 3432, pp. 1-24.
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Bocca, B., Forte, G., **Pisano, A**., Farace, C., Giancipoli, E., Pinna, A., Dore, S., Madeddu, R.
A pilot study to evaluate the levels of aqueous humor trace elements in open-angle glaucoma
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PUBLICATION STAGE: Final
Quartile: Q1; Impact Factor (JCR): 3.24

Farace, C., **Pisano, A**., Griñan-Lison, C., Solinas, G., Jiménez, G., Serra, M., Carrillo, E., Scognamillo, F., Attene, F., Montella, A., Marchal, J.A., Madeddu, R.
Deregulation of cancer-stem-cell-associated miRNAs in tissues and sera of colorectal cancer patients
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DOCUMENT TYPE: Article
PUBLICATION STAGE: Final
Quartile: Q1;

Oggiano, R., **Pisano, A**., Sabalic, A., Farace, C., Fenu, G., Lintas, S., Forte, G., Bocca, B., Madeddu, R.
An overview on amyotrophic lateral sclerosis and cadmium
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HEAVY METALS IN SPUTUM OF IPF PATIENTS, A PUTATIVE ROLE IN DISEASE ETIOLOGY

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