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Study of the molecular mechanisms by which non-coding RNAs (IncH19 and miR-675) control tumor progression and resistance to drug therapy in colorectal cancer

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Abstract

Colorectal cancer (CRC) is one of the most common malignant tumors of the human gastrointestinal system, the third most frequently diagnosed cancer and the second most deadly worldwide, with almost one million deaths annually. The high mortality rate is mainly due to the increasing events of primary or acquired resistance to conventional chemotherapy.
Nowadays, advances in understanding the pathophysiology of CRC have increased the variety of therapeutic options for both local and metastatic diseases, leading to targeted personalised medicine approaches. New therapeutic options are increasingly emerging, such as immunotherapy, non-coding RNA-based anti-tumor therapies or epigenetic therapies based on the use of anti-cancer epigenetic compounds, such as Histone Deacetylase Inhibitors (HDACis).
Accumulating evidence strongly indicates that non-coding RNAs (ncRNAs) are aberrantly expressed in different cancer types and play a crucial role in numerous key biological processes, including drug resistance.
A more precise understanding of the molecular mechanisms of action of the ncRNAs and their specific downstream targets could be advantageous in order to improve therapeutic strategies and overcome chemoresistance events.
This PhD project aims to investigate the role of long non-coding RNA H19 (lncH19) and its intragenic microRNA, miR-675, on the control of tumor progression and chemoresistance in CRC cells, with the ultimate goal of identifying new targets and therapeutic strategies to enhance conventional therapy.
Interestingly, for the first time to our knowledge, our data reveal a dual role of the lncRNA H19 and its miRNA, as both therapeutic targets and as putative prognostic biomarkers.
Indeed, our data demonstrated that lncH19 enforces CRC cell resistance to 5-Fluorouracil (5-FU) especially under chronic hypoxic conditions, through its intragenic miRNA; on the other hand, its expression seems to be functional for the anti-tumor activity of the epi-drug as for the HDACi ITF2357.
Specifically, in the first part of this project we demonstrate that under hypoxic stimulation, lncH19 gives rise to miR-675 which, in turn, inhibits caspase-3 expression. The inhibition of miR-675-5p in combination with 5-FU treatment, enhances the pro-apoptotic effects of the chemotherapeutic drug and overcomes the hypoxia-induced drug resistance. Our data suggest the use of AntagomiR-675-5p as an adjuvant to drug treatment.
In the second part of the project, we found that lncH19 can contribute to the antitumor activity of the HDACi ITF2357. Our data demonstrated that H19, after ITF2357 administration promotes TP53 stabilization by acting as an endogenous competitive sponge (ceRNA) for miRNAs targeting pro-apoptotic genes. Furthermore, we provided evidence that ITF2357 is efficacious in colon cancer model overexpressing the lncH19, and it can overcome the 5-FU resistance.
Overall, the data obtained from the following PhD project unveiled new mechanisms of action by which the lncH19 affects CRC and suggested the use of lncH19 as a putative biomarker to assess the outcome of therapy in patients with 5-FU-resistant CRC.

CHAPTER 1

Colorectal cancer

1.1 Ethology and pathogenesis of CRC

Colon cancer (CRC) is a heterogeneous disease caused by environmental, genetic and biochemical factors [1]. Its development is specifically related to the hyperproliferation of epithelial cells of the colon mucosa [2].
Generally, CRC originates from the non-cancerous proliferation of healthy mucosal epithelial cells, which are continuously renewed. Outgrowths, called "polyps", are then formed, which may grow progressively for 10-20 years before becoming malignant [3, 4]. The most typical form presents as an adenoma or polyp originating from granule cells, producing mucus that lines the large intestine [5]. Colorectal adenocarcinoma, which develops from such polyps, accounts for 90-96% of all CRC variants [4, 6]. In addition to colorectal adenocarcinoma, which is the most common, there are other types of colorectal cancers that can be identified based on their histology. These include:

- **Squamous cell carcinoma**, a very rare type of cancer (about 1% of cases), which develops from the flat cells lining the inner surface of the colon or rectum.
- **Neuroendocrine carcinoma** develops from the colorectum's neuroendocrine cells, which produce hormones and other substances that regulate the endocrine system.
- **Stromal tumors** or **GISTs** (Gastrointestinal Stromal Tumors), which develop from the stromal cells of the intestine, which are responsible for the formation of connective tissue and blood vessels.
- **Lymphomas** from the lymphoid tissue of the intestine.
- **Melanomas** from the melanocytic cells of the intestine.

Genetically, two main forms of CRC can be distinguished:

- **Sporadic**, occurring in individuals without a positive family history or genetic genomic alterations due to acquired somatic genetic mutations or epigenetic alterations induced by modifiable risk factors [7]. Sporadic cases comprise 60-65% of all CRC cases [8] and generally affect patients older than 60 years.
- **Familial**, occurring mainly in individuals with a positive family history when at least one member of the same family has been or is affected by CRC, especially those younger than 50 years of age [9]. Approximately 35-40% of patients with CRC show susceptibility to inherited components [10]. Approximately 7-10% of all CRCs cases are due to inherited mutations in known genes and include Hereditary Non-Polyposis CRC (HNPCC or Lynch Syndrome, caused by germline alterations in DNA

mismatch repair genes, MMR), Familial Adenomatous Polyposis (FAP, an autosomal dominant syndrome caused mainly by inherited mutations in the Adenomatous Polyposis Coli gene, APC), Attenuated Familial Adenomatous Polyposis (AFAP), MUTYH-Associated Polyposis (MAP), APCI 1307K, familial polyposis or Peutz-Jehger syndrome (PJS), Hyperplastic polyposis (HPP), juvenile polyposis (JPS), and hereditary polyposis [11, 12].

The development of CRC is a classic example of multistep pathogenesis, characterized by the progressive accumulation of inherited or spontaneous mutations at oncogenes or tumor-suppressor genes [13].
The acquisition of these mutations is a slow process and, as a result, the development of invasive CRC often takes decades [14]. The mutations found in the sporadic form are the same as those found in the familial form and appear to follow a standard sequential pattern, allowing certain mucosal cells a selective advantage and promoting hyperproliferation and ultimately carcinogenesis [15]. Of course, CRC progression is divided into 4 stages: i) initiation; ii) promotion; iii) progression; and iv) metastasis [16].
The first mutagenic event, termed "gatekeeping", usually involves the tumor-suppressor gene encoding for the APC protein. This initial event causes a disruption of the cell cycle, increasing cell proliferation and leading to hyperplasia. Subsequently, DNA hypomethylation events contribute to the formation of class 1 adenoma, which is a benign neoformation characterized by high cell proliferation. At this early stage, an activating mutation occurs in the Rat Sarcoma oncogene (RAS), leading to the formation of class 2 adenomas. These adenomas not only show cell hyperplasia but also signs of dysplasia. The next mutation causes loss of function of the Deleted in Colon Cancer (DCC) gene, resulting in a class 3 adenoma, in which cells further lose their differentiated morphology. Finally, another mutation causes the loss of function of tumor protein 53 (p53) and is responsible for the transition from a benign tumor to an in-situ tumor [14]. Tumors in situ are polyps that have not yet invaded the wall of the colon or rectum and thus have not yet extended beyond it. Following the loss of function of other tumor-suppressors and the acquisition of function of other oncogenes, this carcinoma in situ acquires the ability to infiltrate blood or lymphatic vessels, forming metastases, thereby invading surrounding tissues and organs and becoming distant tumors [17, 18].
However, one study reported that mutations in all three genes were found in only 7% of CRCs, suggesting that other genes may be involved in the tumorigenic process [19].
To date, it has been extensively documented that there are three genetic and epigenetic aberrations implicated in colorectal carcinogenesis: i) chromosomal instability (CIN), ii) microsatellite instability (MSI), and iii) CpG island methylator phenotype (CIMP) [10, 20]. The most common aberration, occurring in 70-85% of sporadic CRCs, is CIN (adenoma-carcinoma sequence), a process that generates structural and numerical alterations in chromosomes, leading to genetic instability and loss of heterozygosity (LOH), more frequently on chromosomes 5q (APC), 17p (p53) and 18q (DCC/SMAD4) [15, 21–23]. Tumors characterized by CIN usually appear as a consequence of a combination of activation of oncogenes (e.g., KRAS, PIK3CA) and inactivation of tumor-suppressor genes (e.g., APC, SMAD4, and TP53) by allelic loss and mutation [13].
Molecular genetic studies have identified a link between CIN and mutations or loss of function of the tumor-suppressor APC [24]. APC negatively regulates the Wnt signaling pathway, as it is part of a complex that promotes β-catenin protein degradation by the proteasome [25]. Loss of function of the APC gene is a common feature of most sporadic colorectal cancers and results in hyperactivation of the Wnt signaling pathway [26].
Disruption of the Wnt signaling pathway leads to disruption of the regulation of proliferation

and differentiation of colonic mucosal cells, with the formation of dysplastic crypts that evolve into adenomas with an increasing degree of dysplasia, due to the loss of other tumor-suppressor genes [27].
In patients with FAP, germline mutation of the APC gene gives rise to a nonfunctional protein, which leads to the accumulation of β-catenin in the cytoplasm. From the cytoplasm, β-catenin migrates into the nucleus inducing the expression of several genes that drive colorectal tumorigenesis [28].
More than 80% of colorectal cancers have mutations in the APC gene, while an additional 5-10% have mutations or epigenetic changes in other components of the Wnt signaling pathway, such as β-catenin, leading to over-activation of the Wnt pathway [13].
The other less frequent gene aberration, occurring in 15% of sporadic CRCs, is attributed to the unstable nature of MSI, characterized by frequent mutations in simple repeated nucleotide sequences caused by loss of MutL Homolog 1 (MLH1) gene expression, resulting in MMR deficiency leading to hypermethylation and subsequent gene silencing [29–31].
Finally, the pathogenesis of CRC may be influenced by CIMP [32]. CIMP induces genetic instability by increasing alterations in the methylation frequency of CpG islands in the promoter regions of tumor-suppressor genes (e.g., the MLH1 gene, implicated in MMR), rendering their expression attenuated or silencing them completely [33, 34].

1.2 Epidemiology of CRC: incidence, mortality and survival

CRC is one of the most common malignant tumors of the human gastrointestinal system, the third most frequently diagnosed cancer, and the second most deadly worldwide, it produces nearly a million deaths annually [1, 10].
The epidemiology of CRC changes significantly among different regions of the world and among different age, sex, and race groups. Several factors contribute to this variability, including exposure to risk factors, demographic variations, as well as genetic susceptibility, genetic mutations, and their impact on both prognosis and response to treatment [35].
Between 2015 and 2019, the average annual overall CRC incidence rate was 33% higher in men than in women, 41.5 per 100,000 and 31.2 per 100,000, respectively. However, the gender disparity varies by age of diagnosis and tumor location [36].
Overall, women live longer than men, and there is a distal to proximal shift in colon cancer with advancing age. In particular, in the United States around 41% of all colorectal cancers develop in the proximal colon, around 22% in the distal colon and 28% in the rectum [37]. In addition, a recent study suggests that one-third of the CRC sex disparity may be attributed to differences in the prevalence of established sex-related biological risk factors, such as the influence of sex hormones [38].
According to the latest statistics, in 2020, CRC accounted for 10% of all cancer cases worldwide and caused 9.4% of all cancer-related deaths globally. The number of new cases of colorectal cancer worldwide is projected to increase to 3.2 million by 2040, due to factors such as population growth, aging, and human development [1].
More than half of new CRC cases and deaths worldwide occur in China, Europe and North America. While CRC incidence and mortality have decreased in some European and North American countries, they continue to increase in China (***Figure 1***); strongly associated with socioeconomic status (SES), changes in lifestyle and diet [39]. An increasing number of studies show that high SES is associated with better survival in CRC; Indeed, patients with CRC and high socioeconomic status have more favourable surgical treatment characteristics than patients with higher socioeconomic deprivation [40–45]. This disparity in economic

development conditions is more pronounced in Asian countries, causing obvious inequalities in treatment capacity/resources and screening infrastructure [46]. Unless Asian countries implement CRC screening, the incidence and mortality rates of CRC will continue to rise, especially with rapid population growth, economic development, westernised lifestyles and increasing ageing [47]. In fact, in China, new cases of CRC diagnosed in 2020 accounted for 28.8% and the number of CRC-associated deaths accounted for 30.6% of all new cases or CRC-related deaths worldwide, respectively [48].
Similar to incidence, the decline in mortality has slowed from about 4% per year in the early 2000s to about 2% from 2012 to 2020. Since 2012, the overall decline in mortality has exceeded that of incidence (2% versus 1.4%) [36], thanks to the understanding of CRC pathophysiology and the improvements of treatments that have partially inhibited tumor progression. However, trends vary widely by age, race, and ethnicity [1].
From the mid-1970s to 2012-2018, the 5-year relative survival rate for CRC increased from 50% to 65% [36]. This remarkable increase is mainly due to i) the possibility of early diagnosis, through routine clinical examinations and screening, ii) more accurate diagnoses, thanks to advances in imaging, iii) improved surgical techniques chemotherapy and radiotherapy [36].
The 5-year relative survival rate for CRC is similar across regions but varies greatly among European countries [49].

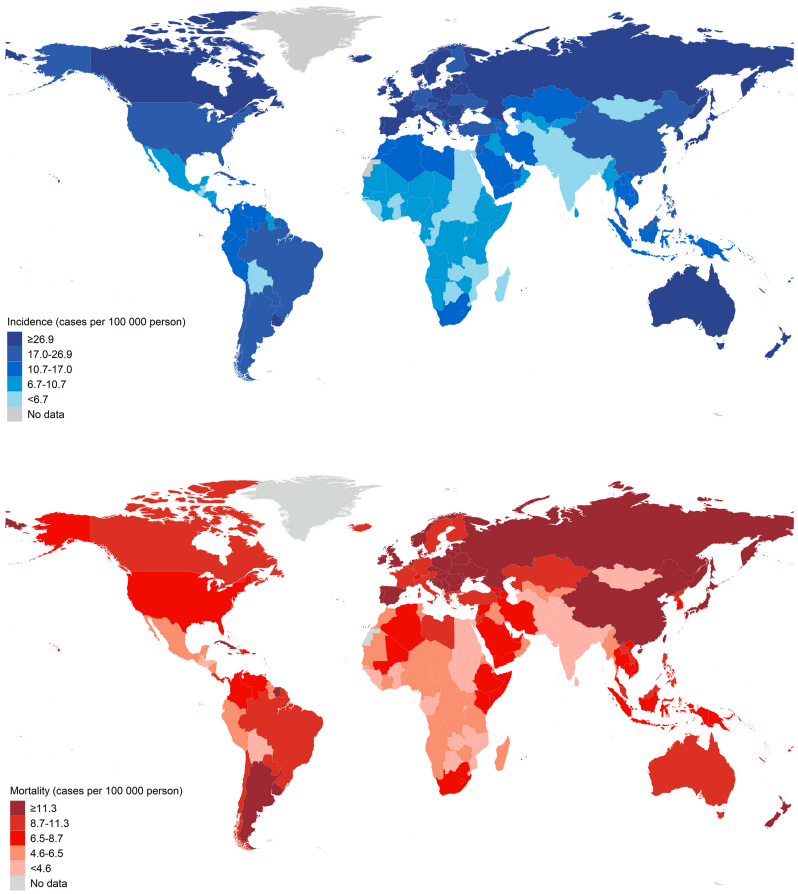

Figure 1. Map showing estimated age-standardized incidence and mortality rates for colorectal cancer in 2020, both sexes (Data are all from GLOBOCAN 2020, https://gco.iarc.fr/today/home) [49].

1.2.1 Early-onset CRC

In contrast to the decrease in the incidence of CRC in the elderly, since the mid-1980s and 1990s, there has been an increase in the incidence of early-onset CRC (EOCRC, defined by the presence of colon cancer in individuals younger than 50 years of age), correlated with increased mortality among young adults aged 40-49 years, which has attracted increasing interest [50]. In fact, cancer trends in young adults are the best indicator of disease progress because they reflect the influence of contemporary exposures versus the long-term cumulative exposures that occur in the elderly [36].
From 2011 to 2019, CRC incidence rates in people younger than 50 years of age and those aged 50-54 years increased by 1.9% per year. The sharp increase in young and middle-aged adults, together with the declining trend in the elderly, has rapidly shifted the incidence of CRC to the younger population [36].
Trends in EOCRC incidence are relatively similar for both sexes but differ according to the site (mainly distal and rectal cancer) and stage (mainly advanced disease) of the tumor [51].
Among European and North American countries, the most significant increase in EOCRC has been observed in the United States, with a rapid increase in Western states associated with the Western diet, but the highest incidence is observed in Southern states and rural areas [52].
The researchers believe that the increase in EOCRC cases is due to a more sedentary lifestyle and suggest that it would be appropriate to lower the screening age of CRC to 45 years to detect cases early in younger adults [3].

1.3 Risk factors and prevention

Several risk factors contribute to the occurrence of CRC. Risk factors can be classified into two main categories: modifiable and nonmodifiable. Modifiable risk factors are those that can be changed or controlled through lifestyle modifications, whereas nonmodifiable risk factors are those that cannot be altered, such as age, gender, and family history.
Recent studies suggest that more than half of CRC deaths cases are attributable to modifiable risk factors [53], mainly due to high exposure to environmental risk factors from a sedentary lifestyle and eating styles projected increasingly toward Westernization [54, 55].
Nonmodifiable CRC risk factors include:

- **Sex, age and ethnicity**
  In 2020, men had a 44% higher incidence rate of CRC compared to women. A major disparity is observed in rectal cancer in comparison to colon cancer. In men, 9.8 cases of rectal cancer and 13.1 cases of colon cancer are diagnosed in 100,000 individuals, 75% and 31% higher, respectively than in women [56]. In the United States, people over the age of 65 are more likely to be diagnosed with CRC than younger people; this is probably related to the age at which screening is suggested. Furthermore, it has been documented that the preferential location of CRC changes with age, especially in black and white patients [57].

- **Genetic risk factor**
  About 35-40% of CRC patients are susceptible to hereditary components [8, 11]. These components include family history, inherited cancer syndromes such as Lynch

syndrome, low-penetrance genetic variations, and other unknown inherited genomic aberrations [11, 12]. People who have a first-degree relative with CRC disease are at a higher risk of developing the disease, with a 2-4 times greater likelihood of getting sick; the same risk remains even beyond first-degree relatives [58].

- **Diseases and therapies that predispose to increased susceptibility to CRC**
  Inflammatory diseases are associated with the risk of developing CRC, i.e inflammatory bowel disease (IBD), ulcerative colitis, Crohn's disease and cystic fibrosis [59, 60]. Moreover, those who have undergone treatment or surgery, including androgen deprivation therapy, cholecystectomy or abdominal radiation, also have an increased risk of developing CRC [61].

Modifiable CRC risk factors include:

- **Obesity and sedentary lifestyle**
  Individuals who are overweight or lead a sedentary lifestyle are at increased risk of CRC; in fact, they have a 25-50% risk of contracting cancer compared with physically active individuals [62]. Obesity and physical inactivity are independent and cumulative risk factors that also reduce survival expectations [63]. A different risk of CRC has been found between overweight men and women; in fact, obese men have a 50% risk of colon cancer and a 20% risk of rectal cancer. In contrast, overweight women have a 20% risk of colon cancer and a 10% risk of rectal cancer. Studies show that in women, early body mass index (BMI) has a greater impact on CRC risk, whereas in men is the late body weight gain [64]. Physical activity may reduce the risk of CRC by inhibiting fat accumulation, suppressing inflammation, and improving intestinal motility and metabolic hormones [65]. Furthermore, at the level of anatomical sites, physical activity is inversely related to the risk of proximal and distal colon cancer, but not rectal cancer [66].

- **Diet and drugs**
  Diet can positively or negatively influence the risk of onset or development of CRC, regardless of obesity [67]. It can exert this function directly through food intake or indirectly through gut microbiome or body weight gain. High consumption of processed foods, red meat and refined carbohydrates, and a diet low in calcium facilitate the inflammatory response and increase the risk of CRC [68, 69], as well as gastric and intestinal cancer. The relative risk of CRC occurrence among those who frequently consume red and processed meat is 1.22% [70]. In contrast, calcium, insoluble fiber, vitamin D, fruits, and vegetables are protective against CRC [55]. In addition, diet and drug use can have a significant impact on the microflora population and intestinal inflammation [71]. For example, the gut microbiota during fermentation of complex food residues releases butyrate, a short-chain fatty acid, which promotes colon health by maintaining mucosal integrity and suppressing inflammation and carcinogenesis [71]. Or the administration of antibiotics may cause dysbiosis of the gut microbiome, which is associated with an increased risk of CRC [72, 73]. Therefore, manipulation of the intestinal microbiome could be a new strategy for the prevention and treatment of CRC [74].

- **Alcohol**
  Alcohol consumption is closely associated with an increased risk of CRC [75]. Individuals who drink 2-3 alcoholic drinks per day have a 20% increased risk of developing CRC, while for those who drink more than three drinks the risk rises to 40%. The correlation between alcohol consumption and its effects on the body is more pronounced

in men, which can be attributed to hormonal changes that affect the metabolism of alcohol [76].

- **Smoking**
  Numerous evidence has shown that smoking predisposes to CRC. In fact, the relative risk of developing CRC in regular smokers is 1.18% [3]. In particular, the intestinal mucosa is damaged by the components of tobacco smoke, causing further genetic or epigenetic alterations [66]. Furthermore, at the level of anatomical sites, smokers appear to have an increased risk of cancer in the proximal colon and rectum, but not in the distal colon [66, 77]. In addition to its effect on CRC onset, smoking is associated with poor CRC survival. Therefore, giving up smoking is linked to better health outcomes and a lower risk of CRC [78].

Variations in CRC incidence and mortality suggest that the disease has a large behavioural component. Therefore, effective prevention measures, such as regular screening, surveillance, and high-quality treatment, can be implemented to reduce the risk of new cases of CRC.
Prevention of colorectal cancer is possible at three different levels:
Primary prevention, involves healthy individuals and consists of reducing exposure to known specific risk factors. This prevention often requires modification of the individual's dietary and physical-behavioural habits. Specifically, it involves regular physical activity, a healthy diet, smoking cessation, and limiting alcohol consumption.
In addition, studies show that long-term intake of nonsteroidal anti-inflammatory drugs (NSAIDs) has chemo-preventive potential against CRC [1, 79].
In particular, CRC patients who routinely use aspirin show less aggressive tumors and longer survival because NSAIDs reduce intestinal inflammation and thus protect against CRC and gastric and small bowel cancers [3].
However, it is still unclear how to adjust the prevention strategy, including the dose and duration of drug intake, to avoid possible adverse effects such as gastrointestinal bleeding or heart attacks [80].
Secondary prevention, is the set of preventive measures designed to detect the disease early and is considered the most efficient in preventing the development of CRC.
The recent introduction of improved screening tests for early detection and new treatment options to eliminate precancerous lesions has greatly influenced CRC incidence and mortality rates [81–83].
Currently, the two-step screening approach generally used in clinical practice [84] involves first performing highly sensitive and minimally invasive tests (FOBT and fecal DNA testing, to reveal molecular markers or blood in the stool) and then, in case of atypical results, performing more invasive validated endoscopic tests, colonoscopy and flexible sigmoidoscopy (FS) to confirm the results [85, 86]. In addition, new screening tests such as CT colonoscopy, capsule endoscopy, and blood or urine biomarkers are being developed [87].
According to the directives of the European Union (EU), the United States and Canada, the best age to start CRC screening is 50 years and screening should be stopped after age 74 [88, 89].
Updated guidelines from the American Cancer Society and the United States Preventive Service Task Force (USPSTF) recommend that in populations at average risk for CRC, regular screening should be performed starting at age 45 years [90]. In contrast, individuals potentially at high risk for CRC due to ulcerative colitis, previous adenomas, or family history should undergo screening for CRC at age 40 (or 10 years before the age of onset in the first-degree relative, in case of family history) and continue until age 74 [49].

Tertiary prevention, based on interventions aimed at preventing recurrences and metastatic growth. Currently, treatments for primary and metastatic colorectal cancer include laparoscopic surgery, radiotherapy, neoadjuvant therapy, biomarker-guided therapy, and chemotherapy [85].
In cases of early diagnosis, the main treatment for CRC is surgery, but this is not effective in cases of metastasis, as in 25% of diagnoses. In these cases, other treatment options such as radiotherapy and chemotherapy are used, including targeted therapies and multi-therapeutic [91, 92].
In conclusion, CRC is a highly preventable disease whose diagnosis can be supported by artificial intelligence-based learning models. Indeed, deep learning models based on CNN (convolutional neural networks) have recently been implemented to detect and characterise the detection rate of adenomas, which help doctors both to read histopathological tissue images and to make more accurate diagnostic and therapeutic decisions [93, 94]. Artificial intelligence-based diagnostic systems can thus significantly improve the readability of medical images, making artificial intelligence technologies very promising in the field of routine CRC screening and treatment [95]. These systems could usher in a new era in CRC prevention programmes focusing on "Leave in-situ" and "Resect and discard" strategies [96].
In addition, machine learning and bioinformatics analysis can help to select and identify more biomarkers of CRC to provide the basis for non-invasive screening [97]. These technologies, along with robotic surgery and new computer-assisted drug delivery techniques, are rapidly gaining popularity for the prevention and treatment of CRC [98].

1.4 Therapeutic strategies and treatment

Nowadays, the progress in understanding the pathophysiology of CRC has increased the variety of therapeutic options for local and metastatic diseases, leading to targeted personalized medicine approaches [80].
The three therapeutic strategies conventionally used for the treatment of CRC include surgery, radiotherapy and chemotherapy [99]. In addition, new treatment options are increasingly emerging, such as immunotherapy [100] or epigenetic therapy based on the use of anti-cancer epigenetic compounds, such as Histone Deacetylase Inhibitors (HDACis) [101].
Surgical therapy: surgical resection has historically been the milestone in the treatment of CRC and still remains the most effective treatment when possible [102, 103]. Minimally invasive laparoscopic resection can accelerate recovery of gastrointestinal function after surgery, reduce hospital stay and does not adversely affect long-term survival [104].
Radiotherapy: uses the action of high-energy radiation (X-rays, gamma rays or charged particles) to eliminate locally present tumor cells through direct and indirect damage (from free radicals produced by radiation), reducing tumor mass and the risk of recurrence.
Chemotherapy: used as neoadjuvant, adjuvant, and palliative therapy. If it is used as "neoadjuvant" treatment, it facilitates the surgeon's task by reducing the size of the neoplastic mass in the patient before surgery. In the case of "adjuvant" therapy, on the other hand, this is given after the removal of the tumor mass to decrease the risk of recurrence. In addition, chemotherapy plays an important role in palliative therapy in case the tumor is at too advanced a stage and inoperable [99].
Among the chemotherapeutic drugs currently in use for the treatment of patients with CRC, according to major guidelines, is 5-Fluorouracil (5-FU), a standard chemotherapy drug

widely used in both adjuvant and palliative chemotherapy [105]. 5-FU is an antimetabolite analogue of pyrimidine that inhibits the function of the enzyme thymidylate synthase, disrupting the synthesis of the nucleoside thymidine and consequently DNA replication and RNA processing [106, 107]. Randomized clinical trials have shown that its administration can result in a significant increase in survival in patients with metastatic carcinoma [108]. To enhance its cytotoxic effect, it is often used in combination with radiotherapy or other anticancer agents, including Leucovorin (an active metabolite of folic acid that can potentiate the interaction of 5-FU with its target enzyme thymidylate synthetase), Irinotecan (administered together with Leucovorin and 5-FU as FOLFOX), and Oxaliplatin (administered together with Leucovorin and 5-FU as FOLFIRI) [100, 109–111].
However, chemotherapy treatments and targeted therapies provide only a limited increase in overall survival for these patients, as their efficacy is increasingly threatened by the rapid evolution of drug resistance events [106, 112].
In fact, despite therapeutic advances, the 5-year survival of CRC patients remains too low [113, 114].
Several studies demonstrated that patients with CRC may develop primary or acquired resistance to 5-FU (90% of patients with metastatic cancer), associated with poor prognosis [112, 115].
Drug resistance developed against conventional therapy is one of the main reasons for chemotherapy failure in cancer, the underlying mechanism of which has not been fully clarified. In fact, resistance to anticancer drugs may depend on several factors, including tumor heterogeneity and changes at the cellular and genetic levels in cancer cells [116]. Chemoresistance can manifest itself through several strategies, including reduced drug uptake due to alterations in enzymes involved in drug metabolism, such as increased thymidylate synthase activity [117] and dysregulation of multidrug resistance (MDR)-inducing drug transporters [118], enhanced DNA repair, and gene amplification [119].
In addition, cellular processes such as apoptosis, autophagy and cell cycle could be inhibited (suppression of apoptosis) or altered in CRC cells, thus affecting the response to 5-FU therapy [106, 120, 121].

1.4.1 ITF2357 (Histone Deacetylase Inhibitor, HDACi)

Recently, the use of histone deacetylase inhibitors (HDACis) as promising anticancer drugs, used alone or in combination with other anticancer drugs and/or radiotherapy, has been gaining increasing popularity [122, 123].
Numerous studies propose the therapeutic use of HDACis for the treatment of several diseases, including metabolic, inflammatory, autoimmune, and neurodegenerative diseases, and not least for the treatment of cancer [124, 125].
HDACis are a well-known class of epigenetic drugs with recognized antitumor activity, targeting aberrant histone deacetylase (HDAC) activity, which is often overexpressed in cancer cells [126, 127]. The action of HDAC inhibitors is to restore or increase the level of histone acetylation, thereby promoting transcriptional activation of tumor suppressors and genes involved in autophagy and apoptotic processes [128–130]. Therefore, inhibition of HDACs represents a valid basis for new anti-tumor therapies [131].
To date, the Food and Drug Administration has approved some HDACis such as vorinostat (SAHA), belinostat (PXD-101), panobinostat (LBH-589), and romidepsin (FK-228) for the treatment of cancer [132]. The HDAC inhibitors SAHA, FK-228 and PXD-101 have been approved for some T-cell lymphomas and LBH-589 for multiple myeloma. Other HDAC inhibitors are in clinical trials for the treatment of hematologic and solid malignancies [124].

In addition, clinical and preclinical studies have shown that these compounds can be used as adjuvants to traditional chemotherapeutics in several types of cancer [133–135].
The results of these studies seem to be promising, for the reversibility of epigenetic changes during cancer development, the effectiveness of targeted epigenetic therapies seems to be of high importance.
Continued efforts are needed to develop HDACis and HDACi-based combination therapies new HDACis with enhanced pharmacokinetics/pharmacodynamics, increased intra-tumor release and class/isoform specificity [136].
Among epigenetic drugs currently being tested in clinical trials and have shown promising anti-cancer potential is ITF2357 (Givinostat). ITF2357 is a potent HDAC inhibitor belonging to the hydroxamic acid class. This compound is currently used in the therapy for the treatment of Duchenne muscular dystrophy, and in clinical trials for Becker muscular dystrophy and juvenile idiopathic arthritis [137–141].
The compound has also revealed a significant anti-tumor action by inducing apoptosis in different tumor models, including leukemia, melanoma, and glioblastoma cells [142–144].
In addition, it has been widely demonstrated that ITF2357 can also act as an adjunct to conventional chemotherapy, increasing sensitivity to demethylating or chemotherapeutic agents such as pemetrexed in lung cancer, doxorubicin in sarcoma cells, and temozolomide in glioma stem cells [145–147].
ITF2357 has recently been reported to exert a targeting effect on oncogenic BRAF in melanoma cells [142] and affect oncogenic BRAF and p53 interplay, thus representing a promising candidate for melanoma-targeted therapy [148].
To date, the only data present in the literature on the effects of ITF2357 in colon cancer are described in a manuscript that discusses the use of the compound for the prevention of colitis-associated cancer in mice [149].
Although, in general, HDACs and HDACis are known to play an important role in the molecular pathophysiology of CRC [150]; to date, there are no data in the literature to our knowledge on the use of ITF2357 for the treatment of human CRC.

CHAPTER 2

Non-coding RNAs and cancer

2.1 Overview of ncRNAs

Recent advances in human transcriptome analysis revealed that the functional products encoded by the genome are not limited only to proteins but include a variety of unique non-coding RNA sequences, called non-coding RNAs (ncRNAs) [151, 152]. Therefore, Francis Crick's central dogma that genetic information only travels from DNA through RNA to protein synthesis has been overturned and the concept of "junk DNA" has been redefined [153].
Almost seventy years ago, with a few exceptions, RNAs were mainly regarded as intermediaries in the protein production process, temporary copies of genetic information (mRNA), components of the ribosome (rRNA) or translators of the codon sequence (tRNA). For many years, proteins have been the main functional end product of genetic information, although the genes that code for them account for less than 1,2% of the genome [151, 153, 154]. Instead, the last decade of scientific discoveries has changed and improved our perception of ncRNAs from "junk" transcriptional products to key regulators that mediate multiple cellular processes, including chromatin remodeling, transcription, post-transcriptional modifications, signal transduction, apoptosis, differentiation and cell metabolism [153].
According to the Encyclopaedia of DNA Elements (ENCODE) transcriptomics project, around 80 per cent of the genome does not code for proteins but is actively transcribed into ncRNAs, whose remarkable and innumerable potential is not entirely clear [154, 155]. Depending on size, shape and position, ncRNAs have been divided into several classes. Based on length they are divided into small non-coding RNAs (<200 bp), such as microRNAs (miRNAs), piwi-interacting RNAs (piwiRNAs), small nuclear RNA (snRNAs), small nucleolar RNAs (snoRNAs) and transfer-RNAs derived small RNA (tsRNAs or tRFs), and large RNAs (>200 bp, circular or linear), including circular RNAs (circRNAs) and long non-coding RNAs (lncRNAs) [156, 157]. In comparison, miRNAs and circRNAs, are ultraconserved transcribed regions, while others, such as lncRNAs, are not generally conserved across species [158, 159].
In detail, the different classes are described below each with its characteristics:

- The discovery of the first **small RNAs** (microRNAs or miRNAs), lineage defective 4 (lin-4) and lethal 7 (let-7) in *Caenorhabditis Elegans*, was more than two decades ago. Today it's known that some RNAs, not coding for proteins, are conserved functional molecules necessary for the embryonic [160], physiological development of organisms and can be expressed in pathological contexts [161, 162]. MiRNAs are a class of small endogenous single-stranded ncRNAs with approximately 22 nucleotides (nt) in length, synthesized from larger RNA transcripts through a complex enzymatic pathway [163]. The miRNAs are transcribed as pri-miRNAs by RNA polymerase II or III, or processed from non-coding RNAs or introns of protein-coding genes

(miRtron) [164]. The pri-miRNAs are then cleaved by the microprocessor complex, formed by the nuclear RNase III enzyme DROSHA and DGCR8, into a stem-loop structure of 70-110 nt, known as the miRNA precursor (pre-miRNA) [165]. The RNase III/DICER/TRBP2 enzyme will cleave the pre-miRNAs transported into the cytoplasm to generate a mature 22-nucleotide double-stranded miRNA duplex. This miRNA duplex includes the miR-3p/miR-5p double-stranded pair, each of which can be selected as functional and recruited by the RNA-induced silencing complex [166, 167]. They mainly regulate gene expression at the posttranscriptional level repressing gene expression through mRNA degradation or translational repression. They act binding the complementary sequence of their target messenger RNAs (mRNA); thereby integrating into the RNA-induced silencing complex (RISC) that contains members of the Ago family of proteins that silence integrated RNAs [168–170]. In addition, miRNAs have been found in the nucleus where they regulate gene expression at the transcriptional level [171, 172].

- **Piwi-interacting RNAs** (piwiRNAs or piRNAs) are a type of small non-coding RNAs that play a biological role by specifically binding to PIWI proteins. They were first identified in *Drosophila*, play a key role in spermatogenesis and germ stem cell maintenance, and are approx. 24-32 nt in length. PiwiRNAs are significantly involved in repression, deadenylation and decay of transposable elements and epigenetic regulation of chromatin, particularly DNA methylation. Recent evidence has revealed that piwiRNAs, in addition to germ cells, are also expressed in cancer cells, where they are involved in the regulation of proliferation, apoptosis and the cell cycle [173–176]. However, their mechanism of action is not entirely clear [156, 177].

- **Small nuclear RNAs** (snRNAs) are small nuclear RNAs of about 150 nt, usually very rich in uracil and ubiquitously expressed [178, 179]. SnRNAs, transcribed by RNA polymerase II or RNA polymerase III, are involved in several important processes such as splicing, regulation of transcription factors or RNA polymerase II and telomere maintenance [180, 181]. A group of snRNAs, known as snoRNAs (small nucleolar RNAs) are located in the nucleolus, play an essential role in RNA biogenesis and guide the chemical modifications of ribosomal and other RNAs. In addition, they can be found associated with specific proteins, forming complexes called snRNPs, which together form the spliceosome [182]. The use of engineered snRNAs has been applied as a potential therapeutic strategy to correct splicing mutations underlying a considerable number of genetic diseases [183].

- **Small non-coding RNAs derived from tRNAs** (tsRNAs or tRFs) are second in order of abundance to miRNAs [184]. They were identified by sequencing high-throughput RNAs from cell lines and are not produced by random degradation of tRNAs but by degradation by specific nucleases [185]. TsRNAs perform several functions, including regulation of gene expression, anti-apoptosis, inhibition of translation, participation in epigenetic regulation, initial virus reverse transcription, promotion of virus replication and cell-cell communication [185, 186]. Some tsRNAs are overexpressed in tumor tissues but underexpressed in normal tissues. Therefore, the relationship between tsRNAs and cancer initiation and development has attracted considerable research attention [187].

- **The circular RNAs** (circRNAs) family differs from other ncRNAs in function and structure and is characterised by a circular configuration with no $5'$ end caps or $3'$ poly(A) tails [188, 189]. This feature, compared to the linear form more common in

ncRNAs, makes circRNAs resistant to degradation by RNase R and thus more stable [190]. These RNAs are expressed in tissue-specific, cell-specific, and developmental stage-specific patterns [191]. Numerous studies show that circRNAs are involved in both physiological and pathological, including cancer, acting as tumor suppressors [192–194] or oncogenes [195–201]. They are involved in the regulation of RNAs or miRNAs, acting as competitive endogenous RNAs, or they can interact with RNA-binding proteins (RBPs), which act as scaffolds for protein-protein interaction [202]. In detail, circRNAs fall into four groups: ecircRNA, ciRNA, EIciRNA, tricRNA. The most studied are the exon-derived circRNAs, called "ecircRNAs"; these are mainly distributed in the cytoplasm, where they act as a sponge for miRNAs, allow protein-protein interaction or, in some cases, can be translated via a cap-independent mechanism [203, 204]. EIciRNAs consist of both exonic and intronic sequences of coding genes, ciRNAs are circular intronic RNAs, enriched mainly in the nucleus where they are involved in gene regulation [205, 206]. Finally, a special group of circular intronic RNAs, the "tricRNAs", are generated during pre-tRNA splicing [207].

- **Long non-coding RNAs** (lncRNAs) are a group of linear RNA transcripts longer than 200 nt [208]. lncRNAs can be transcribed from the exon, intron, intergenic region or $5'/3'$-non-translational regions and fold into complicated secondary structures, which facilitate their interactions with DNA, RNA and proteins [209]. Based on their genomic context, lncRNAs can be divided into five classes: (I) promoter-associated lncRNAs, (II) enhancer-associated lncRNAs, (III) antisense natural transcripts, (IV) gene body-associated (sense) lncRNAs, and (V) long intergenic ncRNAs [210]. With the advancement of next-generation sequencing technologies, several recent studies illustrate the diversity of function of lncRNAs. This diversity may be due to differences in their mechanisms of action, spatiotemporal expression, and/or abundance, which may vary according to the particular cell or tissue type [211]. The lncRNA genes are very similar to protein-coding genes, have marks in their promoters or enhancers, are transcribed by RNA polymerase II, undergo splicing at canonical splicing sites, and are often polyadenylated [212, 213]. Several studies have shown that lncRNAs regulate gene expression at multiple levels through multiple mechanisms of expression, including epigenetic (i.e., X chromosome silencing, genomic imprinting and participation in epigenetic chromatin modulation) transcriptional (activation or inactivation of transcription modulation of transcription factors to bind to promoters) and post-transcriptional (splicing, mRNA turnover, translation, and RNA interference), through interaction with other biomolecules, such as proteins, DNA regulatory regions, and miRNAs; in this way, they function as scaffolds for the regulation of protein-protein interactions and related downstream signaling pathways [214–216]. In addition, the properties of each lncRNA change depending on intracellular localization [208]. At the nuclear level, lncRNAs control gene expression in cis or trans by interacting with transcriptional coregulators and chromatin remodeling complexes or by preventing the binding of transcription factors; also, by interacting with RNA-binding proteins, they can control RNA splicing [214]. At the cytoplasmic level, lncRNAs bind to various partner proteins, thereby regulating RNA stability, degradation, and translation; in addition, they can act as sponges for microRNAs (Competitive endogenous RNAs or ceRNAs), preventing their action and thus targeted inhibition of target mRNAs, as demonstrated in numerous cancer models [217–219].

Non-coding RNAs influence each other. High-throughput deep sequencing of transcriptomes shows that some lncRNAs encode miRNAs or snoRNAs, and some snoRNAs can encode piwiRNAs, regulating their expression as precursors [217].
In addition to regulating mutual gene expression, ncRNAs perform multiple interconnected functions, which is why it has become increasingly difficult to study their function in isolation.
Like miRNAs, some ncRNAs target the mRNAs of many different other genes (and each gene's mRNA may be targeted by multiple miRNAs or piwiRNAs), thus creating a complicated and intricate gene regulatory network [217, 220, 221].
To add further complexity, miRNAs also functionally interact with other ncRNA species, such as circRNAs and lncRNAs, regulating their stability. In addition, it has been reported that interactions between miRNAs and lncRNAs trigger the decay of target lncRNAs and play important roles in the regulation of target genes [153]. Reciprocally, lncRNAs and circRNAs regulate the abundance of available miRNAs by acting as ceRNAs [202, 222–224].
The highly complex nature of some ncRNA interactions supports their role as key regulators in important cellular programs. Perturbations of these interactions have widespread consequences that affect cell fate and are common in cancer.

2.1.1 NcRNAs in cancer

NcRNAs are known for their ability to regulate gene expression through different mechanisms in physiological and pathological developmental contexts. Alterations in their expression contribute significantly to the formation and progression of human malignancies including cancer [216, 225, 226]; in fact, they have been identified as both oncogenic factors and tumor suppressors in several cancer types [151, 156, 227, 228]. The most studied in tumor are lncRNAs and miRNAs, with increasing evidence of their dual function, oncogenic or oncosuppressive, depending on the context in which they are found (***Table 1***).

ncRNAs	**Cancer types**	**Function**	**Description of the mechanisms of action of cancer-related ncRNA**	**References**
Lnc LINC00261	Lung	Tumor suppressor	Active DNA damage response and block proliferation	[229]
Lnc LINC00617	Breast	Oncogene	Induces EMT and metastasis through regulation of the SOX2 stemness factor	[230]
Lnc LINC00959	Colon	Tumor suppressor	Suppresses migration and invasion	[231]
Lnc LINC01138	Liver	Oncogene	Promotes proliferation, invasion and metastasis	[232]
Lnc LINC01271	Breast	Oncogene	Promotes metastasis	[233]
Lnc ANRIL	Bladder, Lung, Liver, Cervical, Stomach	Oncogene	Interacts with PRC2 and CBX7	[234]
Lnc ARLNC1	Prostate	Oncogene	Interacts with the mRNA encoding AR, a nuclear receptor, to promote oncogenic AR signaling, proliferation, and suraval	[235]
Lnc ARSR	Renal	Oncogene	Interacts with transcriptional coactivator YAP and acts as a ceRNA for miRNAs that target RTK transcripts	[236, 237]
Lnc BANCER	Stomach, Skin	Oncogene	Promotes proliferation and metastasis via regulation of NF-kB1, p21, MAPK pathways	[238, 239]

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ncRNAs	Cancer types	Function	Description of the mechanisms of action of cancer-related ncRNA	References
Lnc CamK-A	Breast	Oncogene	Interacts with and controls activity of kinases that modulate calcium-induced NF-KB signaling, leading to remodeling of the tumor microenvironment	[240]
Lnc CCAT1	Colorectal, Esophageal	Oncogene	Interacts with transcription factors (e. G. , SOX2, p63) to activate expression of genes involved in increasing proliferation and decreasing apoptosis	[241, 242]
Lnc CCAT2	Colon, Sophagus, Stomach, Breast, Colon	Oncogene	Elevates chromosomal instability and promote proliferation and invasion. Enhances Wnt signaling pathway via TCF7L2 interaction	[243–245]
Lnc CRNDE	Brain, Leukemia, Colon	Oncogene	Promotes proliferation and invasion. Negatively regulated by insulin and insulin-like growth factors	[246, 247]
Lnc CTBP1-AS	Prostate	Oncogene	Splicing factors to promoter of a nuclear receptor corepressor to decrease its expression, leading to increased oncogenic AR activity	[248]
Lnc DILA1	Breast	Oncogene	Promotes proliferation and multiple drug resistance	[249]
Lnc DILC	Liver	Tumor suppressor	Suppresses stemness	[250]
Lnc DSCAM-AS1	Breast	Oncogene	Interacts with proteins of the hnRNP complex involved in RNA processing and mediates proliferation, invasion, and metastasis	[251]
Lnc EPIC1	Breast	Oncogene	Interacts with MYC transcription factor and increases its activation of target genes, leading to enhanced cell cycle progression	[252]
Lnc ERINA	Breast	Oncogene	Promotes cell-cycle progression	[253]
Lnc FAL1	Ovarian, Breast	Oncogene	Stabilizes components of PRC1 chromatin modifying complex to mediate expression of genes involved in proliferation and suraval	[254]
Lnc GAS5	Breast, Prostate, Lung	Tumor suppressor	Encodes glucocorticoid response element	[255]
Lnc GUARDIN	Lung	Oncogene	Sustains genomic stability and prevent apoptosis and senescence	[256]
Lnc H19	Breast, Brain	Oncogene	Promotes stemness and angiogenesis	[257, 258]
Lnc H19	Breast, Lung, Pancreas, Stomach, Bladder, Prostate, Colon, Skin	Tumor suppressor	Cancer metastasis tumor suppressor and generates miR-675	[259]
Lnc HAS2-AS1	Brain	Oncogene	Promotes invasion	[260]
Lnc HCP5	Stomach	Oncogene	Sequesters miR-3619-5p and upregulates PPARGC1A, which Induces stemness and drug resistance	[261]
Lnc HOTAIR	Esophagus, Stomach, Colon, Liver, Lung, Breast, Ovarian, Bladder, Prostate, Glioma, Melanoma	Oncogene	Recruits PRC2, LSD1/CoREST/REST chromatin modifying complexes, scaffolds transcription factors at target promoters metastasis and proliferation	[262–267]
Lnc HULC	Liver, Pancreas	Oncogene	Modulates abnormal lipid metabolism through miR-9-mediated RXRA signaling pathway	[268, 269]
Lnc HUR1	Liver	Oncogene	Promotes proliferation	[270]

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ncRNAs	Cancer types	Function	Description of the mechanisms of action of cancer-related ncRNA	References
Lnc LET	Liver, Colorectal	Tumor suppressor	Interacts with and destabilizes a dsRNA binding protein (NF90), a key factor involved in regulation of HIF-1a levels and cell invasion	[271]
Lnc lincRNA-ATB	Liver, Breast, Colon, Pancreas	Oncogene	MiR-200 family sponge. Upregulates ZEB1 and ZEB2	[272]
Lnc lincRNA-p21	Colon	Tumor suppressor	Enhances p21 activity	[273]
Lnc lincRNA-ROR	Breast	Oncogene	Competitive endogenous RNA for miR-145	[274]
Lnc LINK-A	Breast	Oncogene	Interacts with kinases that control HIF1α activity, glycolysis, enhances degradation of tumor suppressors	[275, 276]
Lnc LINP1	Ovarian	Oncogene	Promotes proliferation and invasion	[277]
Lnc MALAT1	Lung, Prostate, Colon, Liver, Breast	Oncogene	Forms molecular scaffolds for ribonucleoprotein complexes in the nucleus. Transcriptional regulator for genes involved in cell cycle	[278–280]
Lnc MALAT1	Breast	Tumor suppressor	Inhibition of a pro-metastatic transcription factor (TEAD)	[280, 281]
Lnc MCF2L-AS1	Colon	Oncogene	Enhances cell proliferation and invasion through crosstalk with miR-874-3p/FOXM1 signaling axis	[282]
Lnc MEG3	Brain, Bladder, Bone marrow, Breast, Colon, Liver, Lung, Prostate	Tumor suppressor	Interacts with the tumor suppressor p53 and regulates its target gene expression	[283, 284]
Lnc MIR22HG	Lung	Oncogene	Promotes cell survival	[285]
Lnc RuPAR	Colon, Stomach	Tumor suppressor	Inhibits tumor progression by down-regulation of protease-activated receptor-1(PAR-1)	[286]
Lnc NEAT1	Prostate, Skin	Oncogene	Mediates oncogenic nuclear receptor (ER) signaling, prevents DNA damage and activation of p53 tumor suppressor	[287]
Lnc NEAT1	Pancreatic	Tumor suppressor	Prevent transformation and proliferation	[288]
Lnc NKILA	Breast	Oncogene	Promotes activation-induced cell death in CTLs, T>1 cells, leading to immune evasion	[289]
Lnc PANDA	Leukemia	Tumor suppressor	Inhibits cell growth	[290]
Lnc PCA3	Prostate	Oncogene	Enhances cell proliferation through regulation of PRUNE2	[291]
Lnc PCAT-1	Prostate	Oncogene	Represses expression of BRCA2 tumor suppressor to impact DNA damage repair	[292]
Lnc PGM5-AS1	Colon	Tumor suppressor	Inhibits proliferation and invasion	[293]
Lnc PRNCR1	Colon, Prostate	Oncogene	Binds to the androgen receptor and enhances ligand-dependent and ligand-independent androgen-receptor-mediated gene activation	[294, 295]
Lnc PTENP1	Liver	Tumor suppressor	Suppresses proliferation and invasion	[296]
Lnc PURPL	Colon	Oncogene	Promotes cell growth	[297]
Lnc PVT1	Breast, Bladder, Colon, Kidney, Pancreas	Oncogene	Activates oncogenic signaling (MYC, STAT3) and represses expression of tumor suppressors (p15, p16)	[298–301]
Lnc PVT1	Breast, Ovarian	Tumor suppressor	Inhibits cell growth	[302]
Lnc ROR	Breast	Oncogene	Elevates multiple drug resistance	[303]
Lnc ROR	Brain	Tumor suppressor	Inhibits proliferation	[304]
Lnc RP11-598D14.1	Liver	Tumor suppressor	Inhibits cell growth, cell survival and transformation	[305]

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ncRNAs	Cancer types	Function	Description of the mechanisms of action of cancer-related ncRNA	References
Lnc SAMMSON	Melanoma	Oncogene	Interacts with and controls subcellular localization of proteins that regulate mitochondrial homeostasis and metabolism	[306]
Lnc SChLAP1	Prostate	Oncogene	Interacts with and antagonizes activity of the SWI/SNF chromatin modifying complex to promote invasion and metastasis	[307]
Lnc SCIRT	Breast	Tumor suppressor	Restrains transcriptional program of tumor-initiating cells	[308]
Lnc TCAM1P-004	Liver	Tumor suppressor	Inhibits cell growth, cell survival and transformation	[305]
Lnc THOR	Lung, Melanoma	Oncogene	Binds IGF2BP1 to stabilize interactions with oncogenic target mRNAs, in turn stabilizing those transcripts and promoting proliferation	[309]
Lnc TRG-AS1	Liver	Oncogene	Promotes proliferation and invasion	[310]
Lnc TRINGS	Lung	Oncogene	Protects cancer cells from necrosis	[231]
Lnc TROJAN	Breast	Oncogene	Promotes proliferation and invasion	[311]
Lnc TSLNC8	Liver	Tumor suppressor	Inhibits cell growth, cell proliferation and transformation	[296, 305]
Lnc TUG1	Bladder, Esophagus	Tumor suppressor	Regulates miR-145. Suppresses epithelial-to-mesenchymal transition and radio-resistance	[312, 313]
Lnc UCA1	Colon, Bladder, Breast, Esophagus, Stomach, Liver, Skin	Oncogene	Regulates CREB	[312]
Lnc URHC	Colon	Oncogene	Promotes proliferation and invasion	[314]
Lnc XIST	Breast, Brain	Oncogene	Promotes proliferation, invasion and inhibit apoptosis	[315, 316]
miR-10b	Breast, Glioblastoma	Oncogene	Targets several transcripts that encode regulators of cell cycle progression, migration, invasion, and metastasis	[317–319]
miR-122	Liver	Tumor suppressor	Targets expression of several genes involved in lipid metabolism, proliferation, and inflammation	[320, 321]
miR-1225-5p	Liver	Tumor suppressor	Inhibits proliferation and invasion	[322]
miR-126	Ovarian	Tumor suppressor	Inhibits proliferation	[323]
miR-1274a	Colorectal	Oncogene	Promotes proliferation and metastasis	[324]
miR-128	Brain	Tumor suppressor	Inhibits proliferation and differentiation	[325]
miR-134-3p	Ovarian	Tumor suppressor	Reduces multiple drug resistance	[326]
miR-136	Brain	Tumor suppressor	Promotes apoptosis	[327]
miR-137	Brain	Oncogene	Promotes proliferation	[328]
miR-137-3p	Colorectal	Tumor suppressor	Inhibits migration	[329]
miR-140	Breast	Tumor suppressor	Inhibits proliferation	[330]
miR-141	Breast, Prostate	Oncogene	Promotes proliferation and metastasis	[331, 332]
miR-142-5p	Pancreatic	Tumor suppressor	Inhibits proliferation	[333]
miR-143	Breast	Tumor suppressor	Inhibits proliferation	[334]
miR-145	Prostate	Tumor suppressor	Inhibits proliferation and invasion	[335]
miR-146a	Leukemia	Tumor suppressor	Alleviates myeloma proliferation	[336]
miR-15/16	Leukemia	Oncogene	Sustains stemness	[337]
miR-155	Lymphoma	Oncogene	Targets SHIP1 transcript, a negative regulator of AKT, to increase proliferation and survival	[338–340]
miR-15a/16	Prostate, Leukemia	Tumor suppressor	Targets several transcripts that encode cyclins, CDKs, and anti-apoptotic proteins, thereby increasing apoptosis and inhibiting proliferation	[341, 342]

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ncRNAs	Cancer types	Function	Description of the mechanisms of action of cancer-related ncRNA	References
miR-185	Leukemia	Tumor suppressor	Impairs survival of drug-resistant cells	[343]
miR-190b	Lung	Tumor suppressor	Suppresses cell growth	[344]
miR-200a	Lung	Tumor suppressor	Represses EMT	[345]
miR-200b-3p	Pancreatic	Oncogene	Sustaining self-renewing	[346]
miR-21	Lung, Lymphoma	Oncogene	Targets transcripts that encode negative regulators of RAS signaling, leading to increased proliferation and decreased apoptosis	[347, 348]
miR-22	Colorectal	Tumor suppressor	Represses invasion	[349]
miR-221	Liver	Oncogene	Targets transcripts of tumor suppressors and cell cycle inhibitors (e. G. , p27, PTEN) to increase proliferation and decrease apoptosis	[350, 351]
miR-26a	Glioma, Leukemia	Oncogene	Targets both tumor suppressor (PTEN) and cyclin (CCND2/E2) transcripts to either increase	[352, 353]
miR-26a	Liver, Colorectal	Tumor suppressor	Suppresses tumorigenesis and decrease proliferation	[354, 355]
miR-29	Leukemia	Oncogene	Promotes proliferation in B cells	[356]
miR-29	Glioblastoma	Tumor suppressor	Suppresses cell growth	[357]
miR-30	Breast	Tumor suppressor	Promotes apoptosis	[358]
miR-31	Lung, Breast	Oncogene	Targets transcripts that encode regulators of RAS, WNT, and TGFβ signaling to increase proliferation, stem cell renewal, and metastasis	[359, 360]
miR-34	Prostate	Tumor suppressor	Reduces stemness	[361]
miR-342-3p	Liver	Tumor suppressor	Inhibits proliferation	[362]
miR-34a	Lung, Prostate, Breast	Tumor suppressor	Targets several oncogenic transcripts encoding cyclins, CDKs, cell adhesion molecules, RTKs, and other non-RTKs	[363–366]
miR-506	Ovanan	Tumor suppressor	Targets SNAI2 transcript to decrease its expression and inhibit migration, invasion, and EMT	[367]
miR-518b	Lung	Oncogene	Promotes proliferation and metastasis	[368]
miR-589	Liver	Tumor suppressor	Suppresses stemness	[369]
miR-592	Colorectal	Oncogene	Promotes proliferation and clonogenicity	[370]
miR-600	Breast	Tumor suppressor	Inhibits stemness	[371]
miR-629	Lung	Oncogene	Promotes proliferation and metastasis	[372]
miR-635	Stomach	Tumor suppressor	Inhibits proliferation and invasion	[373]
miR-675	Colorectal, Bladder, Gastric	Oncogene	Regulates tumor suppressor RB, p53 and RUNX1	[374–376]
miR-675	Prostate, Lung	Tumor suppressor	Represses tumor progression and metastasis by targeting TGFBI or GPR55	[377, 378]
miR-7	Breast	Tumor suppressor	Inhibits cell growth	[379]
miR-766	Breast	Oncogene	Promotes proliferation, chemoresistance, migration and invasion	[380]
miR-876-5p	Stomach	Tumor suppressor	Inhibits proliferation and invasion	[381]
miR-93-5p	Liver, Stomach	Oncogene	Suppresses senescence	[382]
miR-99	Leukemia	Tumor suppressor	Suppresses stemness	[383]
let-7	Breast, Ovarian	Oncogene	Sustains self-renewing or multiple drug resistance	[384, 385]
let-7	Lung	Tumor suppressor	Targets several transcripts that encode oncogenes, including RAS, leading to decreased cell cycle progression and proliferation	[364, 386, 387]

Table 1. The most well-known lncRNAs and miRNAs that, based on experimental evidence, exhibit oncogenic functions, tumor suppression functions or both depending on the context.

A deeper understanding of the complex networks of interactions coordinated by ncRNAs would provide a unique opportunity to design better-personalized therapies.
Moreover, many ncRNAs can be released from tumor cells in free-circulating form, bound to lipoproteins or carried by extracellular vesicles into biologic fluids [388, 389]. This has directed the scientific community toward their study as biomarkers for tumor diagnosis, or for prediction of survival, metastasis, or response to therapy [390].
Also, in this case the most studied concern lncRNAs and miRNAs, listed in ***Table 2***.

ncRNAs	Cancer types	Biomarkers types	Source materials	References
Lnc ARSR	Renal	Prognostic for overall survival, recurrence-free survival and prognostic for progression-free survival	Tissue, blood	[236, 237]
Lnc CCAT1	Colorectal	Prognostic for overall survival, cancer-specific survival, and recurrence-free survival	Tissue	[391, 392]
Lnc CCAT2	Colorectal	Prognostic for overall survival and recurrence-free survival	Tissue	[392]
Lnc CamK-A	Breast	Prognostic for overall survival and recurrence-free survival	Tissue	[240]
Lnc EPIC1	Breast	Prognostic for overall survival	Tissue	[252]
Lnc FAL1	Ovarian	Prognostic for overall survival	Tissue	[254]
Lnc H19	Gastric, Gallbladder	For diagnosis and poor prognosis	Tissue, blood	[393, 394]
Lnc HOTAIR	Pancreatic, Esophageal, Ovarian, Breast, CRC	For diagnosis, prognostic for overall survival and metastasis-free survival	Tissue, blood	[263, 395–399]
Lnc HOTTIP	Liver	Prognostic for overall survival	Tissue	[400]
Lnc HULC	Liver	For diagnosis	Tissue, blood	[401, 402]
Lnc LINK-A	Breast	Prognostic for progression-free survival	Tissue	[275]
Lnc LUCAT1	Breast, Liver	Prognostic for overall survival	Tissue	[403]
Lnc MALAT1	Lung, Prostate	For diagnosis and prognostic for overall survival	Tissue, blood, urine	[404, 405]
Lnc NEAT1	Prostate	For diagnosis, prognostic for metastasis-free survival and cancer-specific survival	Tissue	[287]
Lnc PCA3	Prostate	For diagnosis	Urine	[406]
Lnc PCAT-1	Prostate	For diagnosis	Tissue	[407]
Lnc PCAT-14	Prostate	For diagnosis, prognostic for overall survival, metastasis-free survival, and cancer-specific survival	Tissue	[408, 409]
Lnc SChLAP1	Prostate	Prognostic for metastasis-free survival and cancer-specific survival	Tissue	[410, 411]
Lnc UCA1	Bladder, Cholangiocarcinoma, Colorectal, Endometrial, Pancreatic.	For diagnosis, prognostic for overall survival and metastasis-free survival	Urine, tissue	[412, 413]
miR-1	Esophageal	For poor prognosis	Tissues	[414]
miR-103a-3p	Colorectal	For diagnosis	Plasma	[415]
miR-10b	Pancreatic	For diagnosis, prognostic for metastasis-free survival and overall survival	Tissue	[416]
miR-124-3p	Glioma	For diagnosis	Serum exosomes	[417]
miR-127-3p	Colorectal	For diagnosis	Plasma	[415]
miR-128-3p	Lung	For diagnosis	Tissue, blood	[418]
miR-1290	Colorectal, Oral, Prostate	For diagnosis, prognostic for overall survival and progression-free survival	Tissue, blood	[419–421]
miR-135a-3p	Ovarian	For poor prognosis	Serum	[422]
miR-143-5p	Colorectal	For poor prognosis	Fresh frozen biopsies	[423]
miR-146a-5p	Colorectal	For poor prognosis	Fresh frozen biopsies	[423]

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ncRNAs	Cancer types	Biomarkers types	Source materials	References
miR-148a-3p	Colorectal	For poor prognosis	Fresh frozen biopsies	[423]
miR-150-5p	Oral, Colorectal	For diagnosis and poor prognostic	Plasma, fresh frozen biopsies	[423, 424]
miR-151a-5p	Colorectal	For diagnosis	Plasma	[415]
miR-155	Gastric, Leukemia	For diagnosis and prognostic for overall survival	Tissues, blood	[425–427]
miR-16	Lung	Prognostic for overall survival	Blood	[428]
miR-17-5p	Colorectal	For diagnosis	Plasma	[415]
miR-181a-5p	Colorectal	For diagnosis and poor prognostic	Plasma, fresh frozen biopsies	[415, 423]
miR-18a-5p	Colorectal	For diagnosis	Plasma	[415]
miR-18b-5p	Colorectal	For diagnosis	Plasma	[415]
miR-196a-5p	Colorectal	For poor prognosis	Fresh frozen biopsies	[423]
miR-19b	Lymphoma	For diagnosis	Cerebrospinal fluid	[429]
miR-21	Colorectal, Prostate, Lung, Leukemia, Breast	For diagnosis, prognostic for overall survival, cancer-specific survival and progression-free survival	Tissue, blood	[430–435]
miR-210	Pheochromocytomas, Paragangliomas, Colorectal	For diagnosis and poor prognostic	Serum, fresh frozen biopsies	[423, 436]
miR-214	Prostate	For diagnosis	Cell lines	[437]
miR-221	Hepatocellular, Prostate	For diagnosis	Cell lines, tissues, blood	[431, 438]
miR-222	Glioma, Oral, Colorectal	For diagnosis and poor prognostic	Serum exosomes, plasma, fresh frozen biopsies	[417, 423, 424]
miR-223-3p	Colorectal, Breast	For diagnosis and poor prognostic	Fresh frozen biopsies, plasma exosomes	[423, 439]
miR-23a-3p	Colorectal	For poor prognosis	Fresh frozen biopsies	[423]
miR-25-3p	Colorectal	For poor prognosis	Fresh frozen biopsies	[423]
miR-27a-3p	Colorectal	For poor prognosis	Fresh frozen biopsies	[423]
miR-296-5p	Pancreatic	For poor prognosis	Tissues, cell lines	[440]
miR-30b-5p	Colorectal	For poor prognosis	Fresh frozen biopsies	[423]
miR-30c-5p	Colorectal	For poor prognosis	Fresh frozen biopsies	[423]
miR-30d-5p	Colorectal	For poor prognosis	Fresh frozen biopsies	[423]
miR-31-5p	Colorectal	For poor prognosis	Fresh frozen biopsies	[423]
miR-33a-5p	Lung	For diagnosis	Tissue, blood	[418]
miR-34a	Lung	Prognostic for progression-free survival	Tissue	[441]
miR-371a-3p	Germ cell tumors	For diagnosis	Serum	[442]
miR-375	Intestinal, Prostate	Prognostic for overall survival	Biopsies, bloof	[420, 443]
miR-423-5p	Oral	For diagnosis	Plasma	[424]
miR-424-3p	Prostate	For poor prognosis	Tissues	[444]
miR-451a	Pancreatic	For poor prognosis	Plasma exosomes	[445]
miR-491-5p	Gastric	For diagnosis	Tissue, serum	[446]
miR-506	Pancreatic, Ovarian, Gastric	Prognostic for overall survival and progression-free survival	Tissue	[367, 447, 448]
miR-92a	Lymphoma	For diagnosis	Cerebrospinal fluid	[429]
miR-92b-3p	Synovial sarcoma	For diagnosis	Serum, cell lines	[449]
miR-944	Cervical	For poor prognosis	Tissues	[450]

Table 2. The most well-known ncRNAs described as biomarkers for different cancer types.

2.2 LncH19 and its miRNAs

Among the first discovered lncRNAs, is lncH19 [451]. It is located on human chromosome 11p15.5 and has a total length of 2.3 kb (containing 5 exons and 4 introns) [452]. Interestingly, exon 1 of lncRNA H19 encodes for the miRNAs miR-675-3p and miR-675-5p, which in turn plays several key roles in different physiological or pathological contexts [453]. H19 is highly expressed maternally in the developing mouse embryo [451, 454, 455]. After birth it is abundant in skeletal muscle [451] and is developmentally regulated and activated very early during muscle cell differentiation [456].
LncH19 has a highly conserved secondary structure, and its function is structure dependent. [457, 458]. The molecular mechanisms by which H19 acts depend largely on the partners with which H19 interacts (***Figure 2***) [459].
Numerous studies have described that lncH19 is generally and primarily found in the cytoplasm, where it regulates protein translation and activity, mediates mRNA degradation and acts as ceRNA by antagonizing miRNAs [217, 460–462]. However, recent studies indicate its expression also at the nuclear level, where it controls chromatin modification proteins to regulate chromatin remodelling, epigenetic markers and gene expression in cis- or trans- or behaves as a splicing regulator [459, 463, 464]. For example, a very recent study showed that lncH19 acts at the nuclear level by promoting DNA damage repair and PARP inhibition resistance by interacting directly with ILF2 [465].
Also at the nuclear level, lncH19 is implicated in gene methylation; the study by Zhou et al, supports the role of H19 as a regulator of SAHH (S-adenosylmocysteine hydrolase), resulting in altered DNA methylation [466].
In adult tissues, lncH19 expression can be restored during the process of regeneration and tumorigenesis, indicating that H19 is probably related to tumor development and progression [467, 468]. Originally, it was assumed that lncH19 was a tumor suppressor because the H19 transcription competes for transcription factor binding to a neighboring oncogene, IGF2 [469–471]. However, subsequent evidence suggested that, although it acts as a tumor suppressor through transcriptional downregulation of IGF2, lncRNA H19 mainly promotes oncogenesis. H19 is believed to act canonically as an oncogene, as it is upregulated in several tumors and is associated with tumor transformation and malignancy [257, 472–476].
Furthermore, poor patient prognosis correlates with H19 overexpression [477–479]. Several recent research have recognized abnormal expression of lncH19 in a variety of human cancers, such as colorectal [473, 480, 481], liver [482, 483], stomach [484], pancreas [485], oesophageal [486], breast [487, 488], lung [489], glioma [490], ovarian [491] and bladder [492].
LncH19 is also a precursor of antisense RNAs, such as the antisense transcript lncRNA 91H, which is overexpressed in breast cancer [493] or HOTS, the tumor suppressor opposite to H19 [494].
Further studies show that H19 plays a function largely dependent on tumor type, stage of tumor growth and level of molecular signalling pathway [476, 495].
Today, H19 is one of the most extensively studied and documented long non-coding RNAs, functioning alone and as a precursor to the intragenic miRNA miR-675, which also shows pro-tumor activity [496, 497].

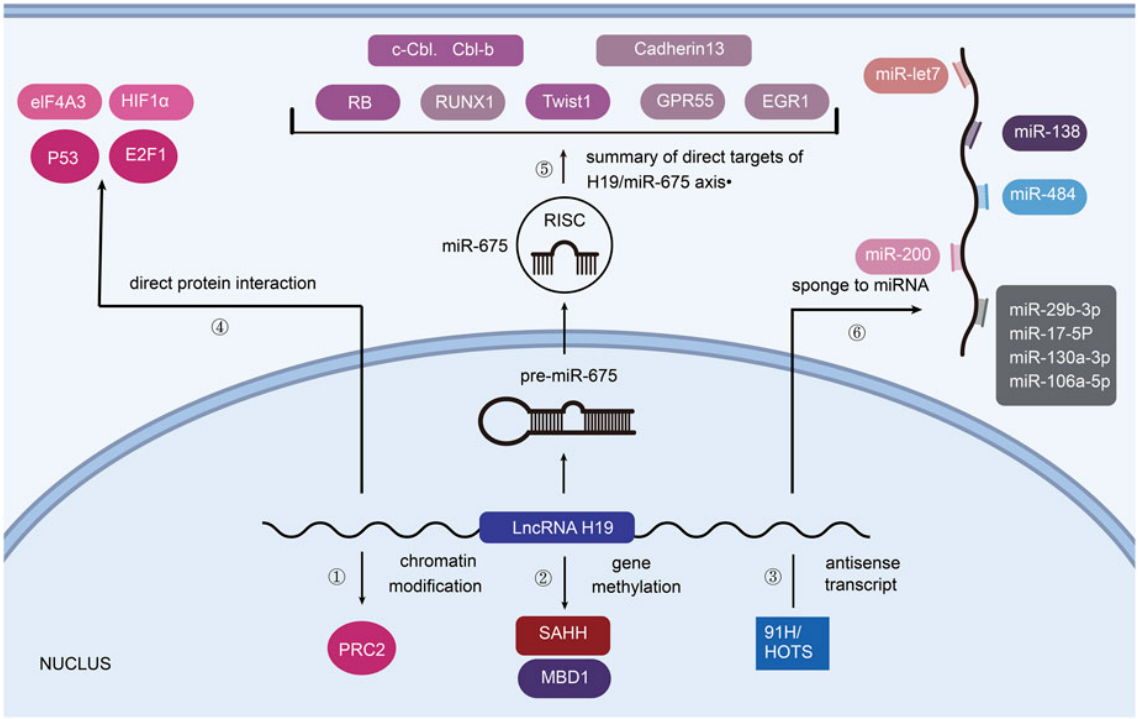

Figure 2. Functions of lncH19 in tumorigenesis [459].

MiR-675 is a highly conserved miRNA, which is synthesized from transcription of exon 1 of its precursor gene H19 by a typical process mediated by DROSHA ribonuclease 3 and DICER [453, 457]; this hosts a miRNA containing a hairpin structure, the loop of which is the most evolutionarily conserved region, and which in its entirety serves as a template for two distinct miRNAs, miR-675-5p and miR-675-3p [458, 498]. The production of miR-675 from its precursor H19 represents a specific gene regulatory mechanism that functions independently of the role of lncH19 [499]. Sometimes miR-675 are the main architects of lncH19 function [458, 467]; they are induced during skeletal muscle differentiation after injury in H19-deficient mice and promote skeletal muscle differentiation and regeneration by targeting Smad anti-differentiation transcription factors [500]. In a very similar way, miR-675 takes the place of lncH19 in metastatic CRC subjected to hypoxic stimulation [480, 496].

Although miR-675 is processed at the expense of H19, there is a positive correlation between the expression of H19 and miR-675. In fact, both are found to be upregulated in many types of cancer, including gastric cancer [376, 501, 502] and colorectal cancer [374, 503]; for this very reason, their relationship remains controversial [504].

The functional roles of miR-675 in tumorigenesis depend on the target gene. Some of the multiple direct or indirect targets of miR-675 are: Retinoblastoma protein (Rb) in colorectal cancer [374], p53 in bladder and colorectal cancer [375], Transforming Growth Factor β (TGFβ1) in prostate cancer [377], Calneuron 1 (CALN1) and Runt Domain Transcription Factor 1 (RUNX1) in gastric carcinoma [376, 501], Cadherin-13 in glioma [505], G Protein-coupled Receptor 55 (GPR55) in non-small cell lung cancer [378], Twist 1 in hepatocellular cancer [506] and Early Growth Response protein 1 (EGR1) in liver cancer [507].

CHAPTER 3

Chemoresistance

3.1 Chemoresistance in cancer

Today, despite numerous advances in cancer research, the most promising strategy for malignancies treatments is chemotherapy [119, 508]. Chemotherapeutic agents can be classified into antimetabolites, alkylating agents, mitotic spindle inhibitors, topoisomerase inhibitors, and other categories based on their mechanism of action [508]. Unfortunately, although the design of new chemotherapeutic agents is growing rapidly, no chemotherapeutic agents have been discovered effective against the advanced stage of cancer, invasion and metastasis. Currently 90% of chemotherapy failures occur during tumor invasion and metastasis due to drug resistance [119, 509].
The acquisition of chemotherapy resistance is a complex process whose underlying mechanism has not been fully elucidated. Cancer drug resistance, in fact, can occur through different strategies, including anticancer drugs inactivation, MDR, cell death inhibition (apoptosis suppression), alteration in drugs' metabolism, epigenetic and drug targets, enhanced DNA repair and gene amplification (***Figure 3***) [119].
In detail:

- Cancer cells are able to inactivate anticancer drugs through specific proteins that allow detoxification of drugs, including enzymes of the glutathione S-transferase (GST) family. Their increased expression in cancer cells, results in reduced drug-induced damage and lethality, as well as apoptosis and increased drug resistance [510, 511].

- The efficacy of anticancer drugs and their activity depend on the absorption and subsequent retention or efflux of the drug in the extracellular environment. Based on this concept, cancer cells are able to survive a wide range of anticancer drugs (MDR) by reducing the uptake or increasing the efflux of the drug to the outside of the cell [512]. For example, overexpression of drug efflux transporters such as the ATP-binding cassette (ABC) offer protection to cancer cells against the negative effects of chemotherapy [513, 514].

- Involved in drug resistance are alterations in the expression and/or activity of multiple apoptosis-related genes and molecules, leading to dysregulation and decreased rates of cell death. In situations of apoptosis, the most commonly reported alterations involve TP53 and its targets, the Bcl-2 family, effector or inducer caspases and mitochondrial caspase (SMAC) [119]. In addition, tumor cells following drug treatment can inhibit their cell death programmes by increasing their autophagy flux and thus selectively removing damaged cytoplasmic components [515, 516]. Recent evidence underlines the close interaction between apoptosis and autophagy in different types of tumors, including CRC. Qian et al. evaluated the interplay between these two processes

in CRC, concluding that autophagy and apoptosis can be induced simultaneously and regulate cell death independently, can mutually induce each other, or that autophagy antagonises apoptosis by preventing the accumulation of damaged DNA and endoplasmatic reticulum stress products [517–519].

- Enzymes play a very important role in the metabolism of chemotherapeutics. Depending on their presence and quantity inside cells, they can modulate the intracellular concentration of the drug, reducing or increasing its activation. The same principle applies to the genes or target proteins of chemotherapeutic agents; mutations or changes in the expression levels of these can vary the effect of the drug on cells [119].
- Epigenetics also plays a key role in the development of chemoresistance. For instance, oncosuppressor genes are often silenced by methylation; conversely, hypermethylation of oncogenes induces their expression. Demethylation of the multidrug resistance gene (MDR1), in tumor cell lines, leads to the acquisition of a multidrug-resistant phenotype and reduces the accumulation of the anticancer drug in tumor cells [520]. Crea et al. collected strong evidence that epigenetic mechanisms trigger resistance to three agents commonly used in CRC (5-FU, Irinotecan and Oxaliplatin) [521].
- It has been described that inhibition of DNA repair systems by chemotherapeutic agents makes cancer cells more sensitive to treatment and increases the efficacy of chemotherapy [119].
- The cell can also resist drug treatment through genetic amplification, increasing the copy number of oncogenes resulting in increased production of related oncoproteins and resistance to chemotherapeutics [522].

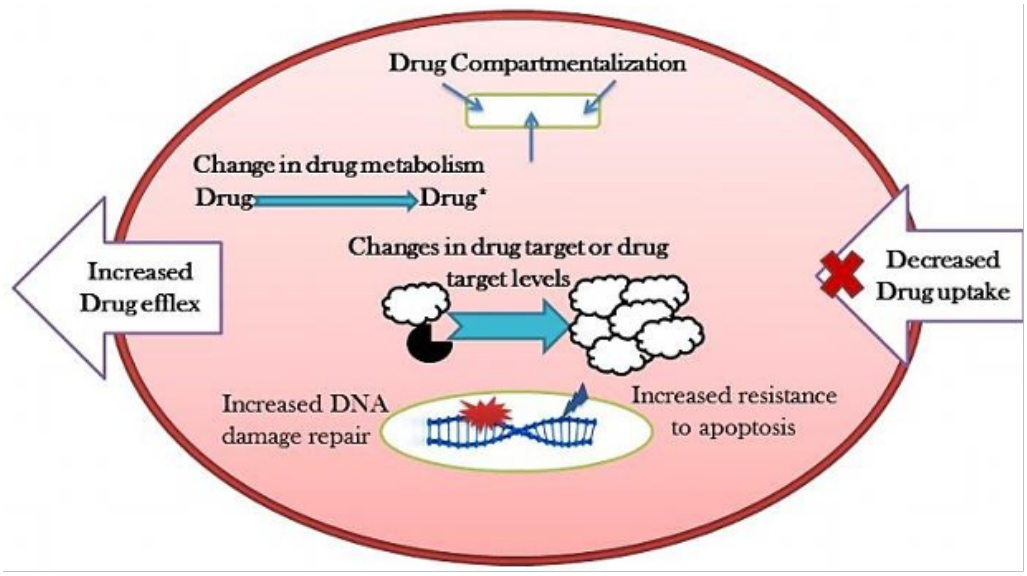

Figure 3. Overview of the mechanisms of drug resistance in cancer cells [119].

Another mechanism that can induce chemoresistance is the low oxygen tension (hypoxia) established in growing tumor mass; it has been associated with a poor prognosis for many cancers including breast [523], hepatocellular carcinoma (HCC) [523] and CRC [524].

3.2 Hypoxia-induced ncRNAs and chemoresistance

Oxygen deficiency is one of the peculiar characteristics of solid tumors, often associated with poor prognosis; tumor cells can develop a hypoxic condition due to inadequate oxygen supply (chronic hypoxia) or transient fluctuations in blood flow (acute hypoxia) [525, 526]. Furthermore, tumor cell metabolism is reprogrammed by this deficiency, readjusting to enable survival, proliferation and ensure tumor progression [527, 528].
Clinically, hypoxia is correlated with metastasis, resistance to chemotherapy and radiotherapy, and worse survival [529–532]. Hypoxia-inducible factor 1α (HIF-1α) and hypoxia-inducible factor 2α (HIF-2α), migrate into the nucleus, heterodimerize with hypoxia-inducible factor 1β (HIF-1β) and transcriptionally activate many genes involved in tumor growth promoting angiogenesis, metabolic switch, autophagy, invasion, and metastasis [528, 533–537]. Despite sharing similar structures, HIF-1α and HIF-2α had highly divergent and even opposing roles in solid tumors under hypoxic conditions. Accumulating evidence in recent years has shown that HIF-1α is associated with chemotherapy failure in many different human cancers, on the other hand, suggests that HIF-2α, at least partly, contributes to chemo/radioresistance through different mechanisms [538]. Therefore, stabilization of HIF-1α induces molecular and phenotypic changes by promoting cell survival, plasticity, motility, and resistance to several anticancer drugs, including 5-FU.
Considering the crucial role of hypoxia and HIF in the context of solid tumors, these have been proposed as possible therapeutic targets in cancer. In recent years, several strategies and drugs have been tested and proposed as therapeutic tools to impair the activity of HIF and related pathways, but only a few of them are in clinical trials presumably due to tolerance limitations, lack of hypoxic selectivity or specificity on the pleiotropism of this transcription factor [539, 540]. In addition to intra-tumor hypoxia, several mechanisms have been reported to contribute to the signalling and regulation of HIF-1/2α, including low molecular weight signal molecules such as reactive oxygen species (ROS), cytokines and growth factors, loss of function of tumor suppressors and increased function of oncogenes. This complexity of regulatory pathways makes the conception and design of HIF-1/2α inhibitors even more complex. Indeed, although some selective HIF-1/2α inhibitors have been proposed, none have been clinically approved. Moreover, other drugs have been shown to indirectly influence the HIF-1/2α pathway, which reflects the connectivity between HIF-1/2α signalling and additional cellular pathways. Consequently, intervention in these ways in cancer leads to an indirect inhibition of HIF activity [541].
To date, further studies are needed to clarify the great complexity of HIF regulation and to identify specific anticancer treatments. The use of HIF-1/2 inhibitors in combination with chemotherapeutics has proven beneficial in several preclinical studies [542]. Hence, there is a need to understand the drug combinations to which the addition of a HIF-1/2 inhibitor will have additive or synergistic effects [541] and the possible molecular mediators through which HIF determines the more aggressive chemoresistant phenotype, in order to identify new and effective therapeutic targets [543].
Over the past few decades, many studies indicated the relevance of non-coding RNAs (ncRNAs) in hypoxia-driven cancer progression and correlated their overexpression with poor prognosis (***Figure 4***) [188, 544, 545].

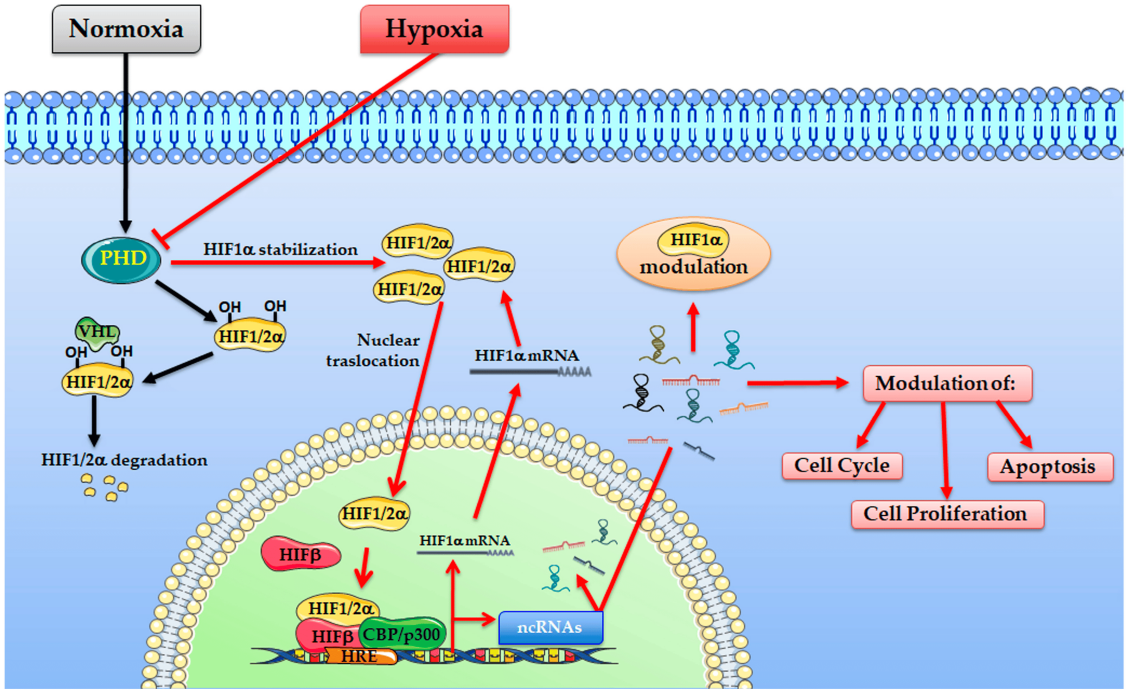

Figure 4. Hypoxia-inducible factor (HIF) complex transcriptionally activates non-coding RNAs (ncRNAs) in response to hypoxia. Under normoxia (black arrows), HIF-1/2α subunits are subjected to hydroxylation by prolyl hydroxylase domain enzymes (PHDs) and other prolyl hydroxylases. Hydroxylated HIF-1/2α subunits are recognized by VHL proteins and targeted for subsequent ubiquitination and proteasomal degradation. Under hypoxia (red arrows), low pO_2 results in HIF-1/2α accumulation, nuclear translocation and dimerization with HIF-β, finally, after recruitment of CBP/p300, the transcription initiation complex binds the promoter of target genes inducing their expression. Among the hypoxia-induced RNAs, the ncRNAs (miRNAs or lncRNAs) will be involved in different pathways, regulating cell proliferation, cell cycle and cell death. Moreover, some of these can regulate HIF itself [188].

Profiling techniques and bioinformatics analysis allowed us to unveil more and more hypoxia-regulated non-coding RNA by the presence of the hypoxia response elements (HREs) in their promoter regions [546]. Moreover, several studies have described hypoxic induction of non-coding RNAs lacking HREs indicating an indirect regulation often involving epigenetic mechanisms; HIF may control non-coding RNAs expression through histone deacetylase activation or affecting miRNA maturation machinery [544, 547].
In addition, recent data demonstrated that HIF-1α can directly regulate circRNAs at the transcriptional level [548–550] and that HIF-induced circRNAs may promote cancer growth as demonstrated in bladder [551]; however, unlike miRNAs and lncRNAs, the mechanisms of HIF-mediated circRNAs expression have been less investigated.
Considering the different mechanisms through which ncRNAs might control tumor growth, these have been divided here into two different groups: (1) the hypoxia-induced ncRNAs that work as HIF effector in promoting cell growth or inhibiting cell death, and (2) the hypoxia induced ncRNAs such as aHIF-1α, linc-ROR, and lincRNA-p21 which directly or indirectly regulate the HIFs proteins (***Figure 5***) [188].

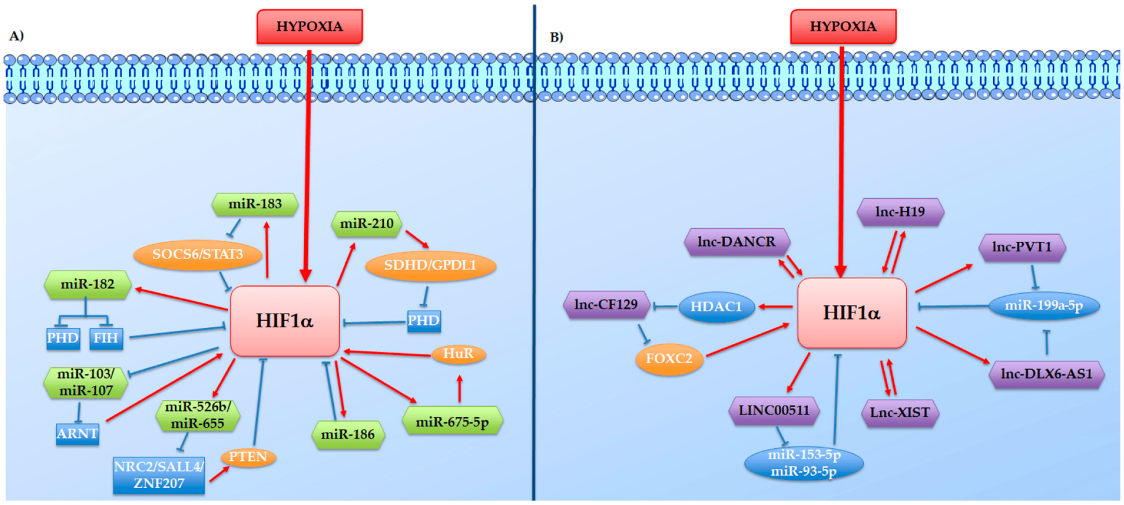

Figure 5. Direct or indirect feedback loops between HIF-1α and hypoxia-regulated ncRNAs [188]. The hypoxia-regulated ncRNAs, HIF-1α, and other co-operators intertwine to form reciprocal feedback loops in both positive and negative manners, represented in the figure respectively with red arrows and blue lines.
A: Reciprocal feedback loops between HIF-1α and hypoxia-regulated lncRNAs.
B: Reciprocal feedback loops between HIF-1α and hypoxia-regulated miRNAs.

3.2.1 HypoxamiRs and chemoresistance

Several studies show that hypoxia induces overexpression or downregulation of various miRNAs, collectively called "hypoxamiRs", which play a key role as emerging mediators involved in the response of tumor cells to the hypoxic microenvironment in a variety of human cancers (***Table 3***) [552–555].
It is important to consider that human tissues may exhibit a different spectrum of responses to hypoxia, including changes in HIF expression that could result from the expression of tissue-specific miRNAs [556]. Indeed, many miRNAs are expressed in tissue- and age-specific models [557]. Since miRNAs act by regulating gene expression and thus downstream protein synthesis, they are ideal candidates for regulating HIF expression during hypoxic situations. Consequently, during the early stages of hypoxia, specific temporal changes in miRNA levels may contribute to the accumulation of HIF-1, while simultaneously keeping HIF-2 and HIF-3 levels stable. On the other hand, in prolonged hypoxia, a change in miRNA expression is observed in order to sustain low HIF-1 activity and maintain high levels of HIF-2 and HIF-3 [556].
Furthermore, it is interesting to note that studies on tumor cells have documented that hypoxia leads to a reduction in the expression of genes involved in miRNA biogenesis, including DICER [558] and DROSHA [559]. At the same time, however, they increased the expression of genes responsible for miRNA function, such as EIF2C4, which encodes a crucial component of the Argonaute 4 RISC complex (AGO4) [558].
A study suggests that hypoxic levels of the great majority of HIF-1α-dependent miRNAs (including miR-210) are also HIF-2α-dependent and that HIF-2α alone regulates the expression of 11 specific miRNAs [560]. Among the main hypoxamiR are miR-210 [561], miR-146a [562], miR-145 [563], miR-382 [564], miR-191 [565], miR-363 [566], miR-224 [567], miR-16 [568], miR-17/20a [569], miR-21/103/107 [553], miR-181b [570] and miR-421 [571] in cancer cells.
Recently, many studies have shown that miRNAs play a key role in the development of MDR) [572–574], such as miR-106a [575], miR-508-5p [576], miRNA-19a/b [577], miR-27a, miR-181a and miR-20b [578].

Besides miRNAs in general, hypoxamiRs may have an impact on several pathways associated with sensitivity or resistance to chemotherapeutic drugs in various cancer types. In particular, among the hypoxamiRs that play an important role in chemoresistance is the hypoxia-induced miR-210-3p, whose upregulation promotes EMT and chemoresistance to Temozolomide (TMZ) in U87-MG glioma cells [579]. Against the same chemotherapeutic agent, overexpression of hypoxia-induced miR-137 inhibited cell invasion and enhanced chemosensitivity of glioblastoma multiforme cells by directly targeting low-density lipoprotein receptor-related protein 6 (LRP6) [580].
Again, HIF-1α-induced miR-421 promotes metastasis, inhibits apoptosis and induces cisplatin resistance by targeting E-cadherin and caspase-3 in gastric cancer [571]. Similarly, Zhang et al. found that hypoxia-induced miR-424 decreases the sensitivity of CRC and melanoma cancer cells (HCT-116 and A375 respectively) to doxorubicin (Dox) and etoposide by inhibiting apoptosis [581]. Furthermore, recently Feng. et al. found that hypoxia-induced up-regulation of miR-27a promotes paclitaxel resistance in ovarian cancer [582].
In contrast, the study by Xu et al. showed that miR-338-5p has a negative correlation with HIF-1α; in a xenograft model, overexpression of miR-338-5p in CRC cells and the HIF-1α inhibitor (PX-478) were able to increase the sensitivity of CRC to oxaliplatin (OXA) [583]. Finally, some miRNAs are able to enhance the sensitivity [584, 585] or resistance [586] of the tumor to the action of chemotherapeutic agents by directly regulating HIF-1α.
Clinically, assessment of the status of these miRNAs may contribute to a detailed understanding of hypoxia-induced resistance mechanisms and/or to the development of future hypoxia-modifying therapies. However, hypoxia-induced miRNAs that may modulate chemotherapy sensitivity by regulating genes involved in hypoxia signalling pathways have not yet been studied in depth [578].
In fact, few data have demonstrated a direct correlation between hypoxia-induced miRNAs and chemoresistance and, in particular, the role of miR-675-5p in hypoxia-induced drug resistance has not yet been studied.

HypoxamiRs	**Cancer types**	**Regulation Hypoxia-mediated**	**Functions**	**References**
miR-100	Bladder	Downregulation	Promotes cell proliferation	[587]
miR-101	Renal	Upregulation	Promotes glycolysis	[588]
miR-103	Colorectal	Upregulation	Promotes hyperproliferation and decreases apoptosis	[589]
miR-107	Colorectal	Upregulation	Promotes hyperproliferation and decreases apoptosis	[589]
miR-137	Glioblastoma	Downregulation	Inhibits cell viability and promotes apoptosis	[580]
miR-145	Bladder	Upregulation	Induces apoptosis	[563]
miR-146a	Leukaemia	Upregulation	Associated with chemoresistance	[562]
miR-16	Lymphomas	Downregulation	Regulates angiogenesis	[568]
miR-17	Leukaemia	Downregulation	Regulates cell cycle	[569]
miR-181a	Gastric	Downregulation	Associated with chemoresistance	[578]
miR-181b	Retinoblastoma	Upregulation	Promotes cell proliferation	[570]
miR-191	Lung, Breast	Upregulation	Promotes cell proliferation and migrations	[565, 590]
miR-199a	Liver	Downregulation	Associated with a worse survival	[591]
miR-204	Liver	Downregulation	Induces tumor cell proliferation	[592]
miR-20a	Leukaemia	Downregulation	Regulates cell cycle	[569]
miR-20b	Gastric	Downregulation	Associated with chemoresistance	[578]
miR-21	Cervical, Gastric, Pancreatic	Upregulation	Promotes cell growth, proliferation and inhibits apoptosis	[593, 594]

Table 3 – Continued on next page

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HypoxamiRs	Cancer types	Regulation Hypoxia-mediated	Functions	References
miR-210	Glioma, Liver, Ovarian, Schwannoma, Neuroblastoma	Upregulation	Promotes/inhibits cell proliferation and apoptosis	[595–599]
miR-224	Gastric	Upregulation	Promotes cell growth	[567]
miR-27a	Gastric, Ovarian	Upregulation	Promotes cell proliferation and chemoresistance	[582, 600]
miR-27a	Gastric	Downregulation	Associated with chemoresistance	[578]
miR-338	Colorectal	Downregulation	Associated with chemoresistance	[583]
miR-33a	Liver	Downregulation	Upregulates tumor cell proliferation	[601]
miR-363	Leukaemia	Upregulation	Regulates hematopoiesis	[566]
miR-382	Gastric	Upregulation	Promotes cell proliferation	[564]
miR-421	Gastric	Upregulation	Inhibits apoptosis and induces chemoresistance	[571]
miR-424	Breast	Upregulation	Inhibits apoptosis	[581]
miR-548an	Pancreatic	Downregulation	Inhibits cell proliferation	[602]
miR-675	Colorectal, Lung, Gastric	Upregulation	Regulates cell cycle, promotes cell proliferation and inhibits apoptosis	[480, 496, 505, 603, 604]

Table 3. Main hypoxamiRs that, based on experimental evidence, play different roles in several cancer types.

3.2.2 Hypoxia-induced lncRNAs and chemoresistance

Accumulating evidence shows that HIF promotes rapid and effective phenotypic changes through the induction of hypoxia-responsive lncRNAs [605]. In fact, hypoxia-induced lncRNAs regulate several biological processes related to tumorigenesis, including tumor growth/proliferation, tumor metabolism, angiogenesis, tumor migration/invasion, apoptosis and chemoresistance [606–609].

At the same time, recent evidence has shown that lncRNAs can regulate the expression or activity of HIF-1α and consequently modulate downstream targets and cellular processes mediated by HIF-1α [610, 611].

Thus, lncRNAs can reciprocally regulate hypoxia signalling by stabilising HIF-1α through several mechanisms, including epigenetics, RNA stability and translation, protein stability and regulation of HIF-1α transcriptional activity (***Figure 6***) [610].

Among the emerging lncRNA regulators of HIF1a is the lncRNA CASC9, which can be induced by HIF-1α and reciprocally promote the stability of HIF-1α [612]; together HIF-1α and CASC9 may form a reciprocal positive feedback loop to facilitate cell proliferation and metastasis in lung cancer [613]. Recently, Cheng-Ning Ma et al. identified hypoxia-induced lncRNA HABON as a regulator of HIF-1α; specifically, the lncRNA HABON interacts with HIF-1α to promote its protein degradation, thereby influencing the transcription of HIF-1α's target genes to exert its effects on hepatocarcinoma cells [614].

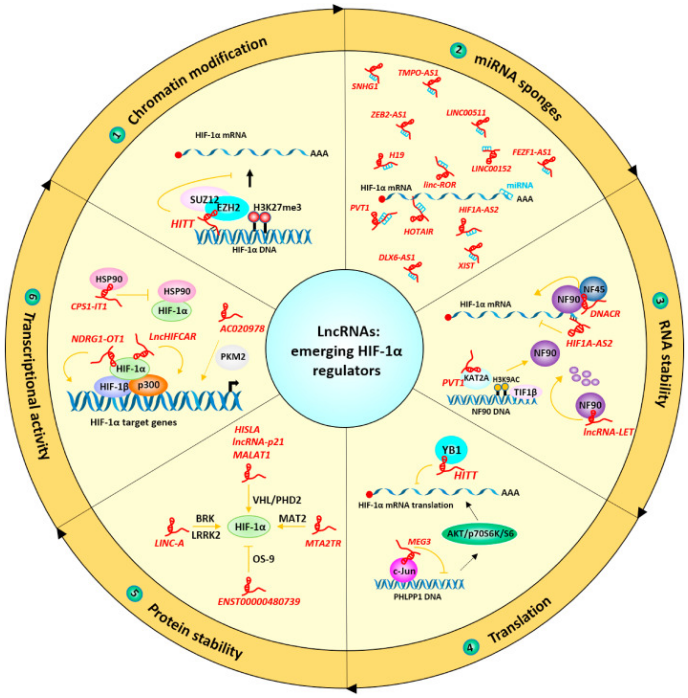


Figure 6. LncRNAs: emerging HIF-1α regulators [610].

The majority of hypoxia-induced lncRNAs have an oncogenic function. However, some hypoxia-induced lncRNAs play an opposing role in tumor proliferation and migration (***Table 4***). As shown by Wang et al., they demonstrated that suppression of HIF2PUT, a HIF-2 α-induced lncRNA, accelerates proliferation of the human osteosarcoma cell line MG63 [615]. Hypoxia-induced lncRNAs that play an oncogenic role include lncRNA HOTAIR, lncRNA-BX111, lncRNA GAPLINC and lncRNA RP11-390F4.3 which, in non-small cell lung cancer, pancreatic carcinoma, gastric carcinoma and breast cancer, respectively, promote metastasis and tumor progression [616–619].
Hypoxia-induced overexpression of lncRNAs MALAT and NEAT also promotes tumor progression by acting as ceRNAs. For example, the lncRNA MALAT competitively bind miR-3064-5p, thereby promoting breast cancer cell proliferation and migration [620].
Similarly, lncRNA NEAT, overexpression of which is induced by HIF-2α, competitively binds miR-101-3p, participating in the progression of non-small cell lung cancer via the miR-101-3p/SOX9/Wnt/β-catenin axis [621].
Using microarray analysis on hypoxia-induced gastric cancer cell lines, Wang et al. identified several hypoxia-responsive lncRNAs in gastric cancer. In particular, they found that an intronic antisense lncRNA named lncRNA-AK058003 was among the most induced lncRNAs upon hypoxia treatment in all examined gastric cancer cell lines [622], data confirmed also in breast cancer [623].
Recent studies have indicated that hypoxia-induced lncRNAs play an important role in chemotherapy resistance, these include lncRNA LUCAT1, lncRNA EMS, lncRNA NORAD and lncH19. Hypoxia-induced ncRNA LUCAT1 confers chemoresistance to CRC cells both in vitro and in vivo. LUCAT1 physically interacts with PTBP1 (Polypyrimidine Tract Binding Protein 1) to modulate the alternative splicing of a set of DNA damage-related genes [624].
Zhu et al. demonstrated that targeted knockout of hypoxia-induced lncRNA EMS and WTAP decreased chemoresistance to cisplatin treatment in a xenograft mouse experimental model. Evidencing the involvement of the EMS/miR-758-3p/WTAP axis in the regulation

of hypoxia-mediated cisplatin resistance in oesophageal cancer [625].
Furthermore, Zhang et al. demonstrated that hypoxia-induced lncRNA NORAD promotes vasculogenic mimicry and resistance to 5-FU by acting as ceRNA of miR-495-3p in hypoxic CRC [626].
Finally, our work demonstrated that lncH19 indirectly, through its intragenic miRNA, miR-675-5p, promotes chemoresistance to 5-FU in CRC under hypoxic conditions [627].
Till now, the specific mechanisms of most hypoxia-induced lncRNAs to regulate cell proliferation and chemoresistance are still unknown and need to be further elucidated in the future.

Hypoxia-induced lncRNAs	**Cancer Types**	**Regulation Hypoxia-mediated**	**Regulatory mechanism**	**Functions**	**References**
Lnc AC020978	Lung	Upregulated HIF-1α direct	Transcriptional regulation	Promotes cell proliferation and glycolysis	[628]
Lnc AK058003	Gastric	Upregulated HIF-1α	Interaction chromatin/epigenetic regulators	Promotes migration and tumor progression	[622]
Lnc BC005927	Gastric	Upregulated HIF-1α direct	Interaction chromatin/epigenetic regulators	Promotes tumor progression	[629]
Lnc BX111	Pancreatic	Upregulated HIF-1α direct	Transcriptional regulation	Promotes cell proliferation	[617]
Lnc CASC9	Nasopharyngeal, Lung	Upregulated HIF-1α direct	Transcriptional regulation and interaction protein or protein complex	Promotes glycolysis, tumor progression and metastasis	[612, 613]
Lnc CRPAT4	Renal	Upregulated HIF-1α direct	Transcriptional regulation	Promotes cell migration	[630]
Lnc DARS-AS1	Myeloma	Upregulated HIF-1α direct	Post-translational modification	Promotes tumorigenesis	[631]
Lnc EFNA3	Breast	Upregulated HIF-1α direct	Protein accumulation	Promotes metastatic dissemination	[632]
Lnc EIF3J-AS1	Liver	Upregulated HIF-1α direct	Sequestration of miRNAs	Promotes cell proliferation and migration	[633]
Lnc EMS	Esophageal	Upregulated HIF-1α direct	Sequestration of miRNAs	Promotes chemoresistance	[625]
Lnc GAPLINC	Gastric	Upregulated HIF-1α direct	Transcriptional regulation	Promotes cell proliferation and migration	[618]
Lnc H19	Multiple Myeloma, Glioblastoma, Lung, Prostate	Upregulated HIF-1α direct and indirect	Transcriptional regulation and interaction protein or protein complex	Promotes migration, invasion, tumor progression and cell dissemination	[472, 604, 634, 635]
Lnc HABON	Liver	Upregulated HIF-1α direct	Transcriptional regulation	Promotes migration cell growth and proliferation	[614]
Lnc HAS2-AS1	Oral	Upregulated HIF-1α direct	Transcriptional activation	Promotes cell invasion	[636]
Lnc HIF1A-AS2	Glioblastoma	Upregulated HIF-1α direct	Transcriptional regulation	Involved in stem cell growth, cell renewal and survival	[637]
Lnc HIF2PUT	Osteosarcoma	Upregulated HIF-2α dependent	Interaction protein or protein complex	Decreases cell proliferation and migration	[615]
Lnc HINCUT-1	Colorectal	Upregulated HIF-1α	Transcriptional regulation	Promotes cell proliferation	[638]
Lnc HOTAIR	Lung, Liver	Upregulated HIF-1α direct	Sequestration of miRNAs	Promotes cell proliferation, migration and glycolysis	[616, 639]
Lnc HOTTIP	Glioma, Lung	Upregulated HIF-1α direct	Sequestration of miRNAs	Promotes migration, invasion and glycolysis	[640, 641]

Table 4 – Continued on next page

Table 4 – Continued from previous page

Hypoxia-induced lncRNAs	Cancer Types	Regulation Hypoxia-mediated	Regulatory mechanism	Functions	References
Lnc LET	Gallbladder, Colorectal, Lung, Liver	Downregulated HIF-1α direct or indirect/ epigenetic (HDAC3)	Transcriptional regulation	Induces cell viability, proliferation and metastasis	[271, 642]
Lnc Linc-01436	Lung	Upregulated HIF-1α direct	Sequestration of miRNAs	Promotes cell migration and invasion	[643]
Lnc Linc-p21	Cervical, Breast	Upregulated HIF-1α direct	Interaction protein or protein complex	Promotes glycolysis	[644]
Lnc Linc-ROR	Liver	Upregulated HIF-1α direct	Sequestration of miRNAs or present within the extracellular vesicles	Promotes cell survival	[645]
Lnc LUCAT1	Colorectal	Upregulated HIF-1α direct	Interaction protein or protein complex scaffold and transcriptional activation	Induces chemoresistance	[624]
Lnc MALAT1	Lung, Liver	Upregulated HIF-1α direct or HIF-2α direct	Transcriptional regulation and sequestration of miRNAs	Promotes cell proliferation, migration and glycolysis	[646–649]
Lnc MTA2TR	Pancreatic	Upregulated HIF-1α direct	Transcriptional regulation	Promotes tumorigenesis	[650]
Lnc NDRG-OT1	Breast	Upregulated HIF-1α	Post-Translational modification	Promotes protein degradation	[651]
Lnc NEAT1	Breast, Lung	Upregulated HIF-2α dependent	Transcriptional regulation and sequestration of miRNAs	Promotes cell proliferation, tumorigenesis and paraspeckle formation	[621, 652]
Lnc NICI	Renal	Upregulated HIF-1α direct	Transcriptional regulation	Involved in glycolysis and cell proliferation	[653]
Lnc NORAD	Pancreatic, Colorectal	Upregulated HIF-1α direct	Transcriptional regulation and sequestration of miRNAs	Promotes migration, tumor progression and chemoresistance	[626, 654]
Lnc NUTF2P3	Pancreatic	Upregulated HIF-1α direct	Transcriptional activation and sequestration of miRNAs	Promotes cell proliferation and tumor progression	[655]
Lnc PCGEM1	Gastric	Upregulated HIF-1α dependent	Transcriptional regulation	Promotes cell proliferation and invasion	[656]
Lnc RAB11B-AS1	Breast	Upregulated HIF-2α direct	Transcriptional regulation	Promotes angiogenesis and metastasis	[657]
Lnc RP11-390F43	Breast, Hypopharyngeal	Upregulated HIF-1α direct	Transcriptional regulation	Promotes migration and tumor progression	[619]
Lnc RUNX1-IT1	Liver	Downregulated HIF-1α indirect/ epigenetic (HDAC3)	Sequestration of miRNAs	Decreases cell proliferation	[658]
Lnc SARCC	Renal	Downregulated HIF-2α direct	Post-Translational regulation	Promotes/Inhibits cell proliferation	[659]
Lnc UCA1	Gastric, Bladder	Upregulated HIF-1α direct	Sequestration of miRNAs	Promotes cell proliferation, tumor progression and drug resistance	[660, 661]
Lnc WT1	Leukemia	Upregulated HIF-1α indirect/ epigenetic (DNA demethylation)	Interaction chromatin/epigenetic regulators	Involved in stem cell function	[662]

Table 4. Main hypoxia-induced lncRNAs that, based on experimental evidence, play different roles several cancer types.

CHAPTER 4

Objectives

To date, it is known that 98% of the human transcriptome encodes for several classes of non-coding RNAs (ncRNAs), whose biological relevance is gradually being recognized, especially in different types of cancer. Several published data strongly indicate that ncRNAs are aberrantly expressed in cancer and are involved in multiple biological processes such as proliferation, differentiation, tumor progression, apoptosis and drug resistance.
Currently, numerous studies suggest that an increasing number of patients with colorectal cancer (CRC) show primary or acquired resistance to 5-Fluorouracil (5-FU), the standard chemotherapy generally used in both adjuvant and palliative chemotherapy.
This resistance is associated with poor prognosis; therefore, it is critical to better understand the molecular mechanism underlying drug resistance by studying the possible involvement of ncRNAs or new drugs, with antitumor activity.
During my PhD I investigated the role of the lncH19 and its intragenic miR-675 in regulating drug responses in CRC, with the ultimate goal of identifying new targets and effective therapeutic strategies to enforce conventional therapy.
To pursue this goal I directed my studies towards two main objectives:

- the first aims to investigate the role of the hypoxia-induced ncRNA miR-675-5p in 5-FU chemoresistance on CRC cells, to identify new and more effective molecular targets for the treatment of colorectal cancer. The data obtained to pursue this goal are described in ***Chapter 5*** and published in the scientific journal BMC Cancer with the title: ***"Mir-675-5p supports hypoxia-induced drug resistance in colorectal cancer cells"*** [627].

- the second aims to investigate the use of an HDACi (ITF2357) in CRC cells investigating its effects on lncH19 expression and activity. The data obtained to pursue this goal are described in ***Chapter 6*** and published in the scientific journal Frontiers in Pharmacology with the title: ***"Long non-coding RNA H19 enhances the pro-apoptotic activity of ITF2357 (Histone Deacetylase Inhibitor) in colorectal cancer cells"*** [663].

CHAPTER 5

"MiR-675-5p supports hypoxia-induced drug resistance in CRC cell"

5.1 Materials and Methods

5.1.1 Cell culture

HCT-116 and SW480 cells (ATCC-LGC Standards S.r.L., Italy) were cultured respectively in McCOY'S 5A medium and RPMI 1640 (Euroclone, UK) supplemented with 10% Fetal Bovine Serum, 1% Penicillin/Streptomycin (10,000 U/mL Penicillin and 10 mg/mL Streptomycin) and 200 mML-Glutamine (all from Euroclone, UK).
Cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C and used at early passages (under 10 passages) for all experiments. The culture medium was changed every 2-3 days, and cells were split at 70–80% of confluence.

5.1.2 Hypoxia assay

To perform hypoxia experiments, cells were seeded on cell culture plates (Sarstedt), maintained for 24 hours in a humidified atmosphere of 5% CO_2 at 37°C, and finally moved into a hypoxic chamber (Stemcell™ Technologies, Voden Medical Instruments spa, Italy) containing 1% O_2 gas mixture for 72 hours, the suitable time to achieve hypoxia-induced drug resistance [664].

5.1.3 MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay

Cell viability was determined by MTT assay following the manufacturer's instructions (Cat. n° M6494, Thermo Fisher®, USA) and the absorbance at 540nm was measured by the Microplate Reader (BioTek Instruments, USA). HCT-116 and SW480 were seeded in quadruplicate respectively at 3×10^4 or 2.5×10^4 cells/cm^2. After 24 hours, the cells were treated with 5 or 10µM of 5-FU (5-Fluorouracil, cat. n° F6627, Sigma-Aldrich, St. Louis, MO, USA) and placed for 72 hours in hypoxic (hypoxic chamber containing 1% O_2 gas mixture) or normoxic conditions.

5.1.4 Transfection

HCT-116 and SW480 cells were seeded respectively at 3×10^4 or 2.5×10^4 per cm^2. The day after, cells were transfected with 3.7 pMoles/cm^2 of miRCURY LNA miRNA Inhibitor hsa-miR-675-5p (Cat. n°339203 YCI0202815-FZA, Qiagen, Germany), or miRCURY LNA miRNA Inhibitor Negative Control (Cat. n°339,203 YCI0202036-FZA, Qiagen, Germany). For cell transfection, HiPerFect Transfection Reagent (Cat. n° 301704, Qiagen, Germany)

was used following the manufacturer's standard instructions. Six hours after transfection, the cells were treated with 10µM of 5-FU in a fresh medium and placed for 72 hours in hypoxic or normoxic conditions. After this time cells were used for MTT assay or protein and RNA extraction.

5.1.5 RNA extraction and Real-Time PCR (qRT-PCR)

Total RNA was extracted using the commercially available TRIzol® RNA Isolation Reagents (Cat. n° 15596026, Thermo Fisher® Products & Kits, USA) according to the manufacturer's instructions. The total RNA concentration was detected with Nanodrop spectrophotometer (Thermo Fisher®, USA). Reverse transcription and qRT-PCR were performed following the manufacturer's instruction by the use of TaqMan™ MicroRNA Reverse Transcription Kit (Cat. n° 4366596, Applied Biosystem™, USA) and TaqMan™ Fast Universal PCR Master Mix. (Cat. n° 4352042, Applied Biosystems™, USA). For probes and oligonucleotides were used Has-miR-675-5p cod. TM002005 and U6 snRNA cod. TM001973 (all from Applied Biosystems™, USA). Hsa-miR-675-5p expression levels were normalized to U6 snRNA and data are presented as $2^{-\Delta\Delta Ct}$.

5.1.6 MirWalk target prediction

The miR-675-5p targets prediction among apoptosis pathway was performed using the tool Target Mining of mirWalk 2.0 database search engine [665].

5.1.7 Wester blotting

HCT-116 and SW480 cells were lysed for 1,30 hours in Lysis Buffer (15 mM Tris/HCl pH 7.5, 120 mM NaCl, 25 mM KCl, 1 mM EDTA, 0.5% Triton X100) addicted with Phosphatase Inhibitor cocktail (Cat. n° 37492, Active Motif, USA). Cell debris was removed by centrifugation at 17.000 g for 15′ at 4 °C and the supernatant, containing protein lysate, was quantified by the Bradford microassay method (Pierce™ Coomassie Plus Assay Kit, Cat. n° 23236, Thermo Fisher Scientific, USA) using Bovine Serum Albumin (BSA, Cat. n° A2153, Sigma-Aldrich, USA) as a standard. A total of 15 µg of protein from each sample was separated using Bolt Bis-Tris gel 4 – 12% (Cat. n° NP0326BOX, ThermoFisher Scientific, USA) and transferred on nitrocellulose membranes with pore size 0.45 µm (Cat. n° GEH10600002, GE Healthcare, USA). The membranes were coloured with 0.1% Rosso Ponceau in 5% acetic acid to evaluate the correct loading and migration of all samples. The membranes were incubated for 1 hour in blocking solution (5% BSA, 20 mM Tris, 140 mM NaCl, 0.1% Tween-20) and overnight with the primary antibodies: anti-Carbonic Anhydrase/CA9 (1:1000, Cat. n° 5648S, Cell Signaling Technology, USA), anti-PARP-1 (1:500, Cat. n° sc-8007, Santa Cruz Biotechnology USA), anti-Cleaved aspase-3 (1:400, Cat. n° 9664S, Cell Signaling Technology, USA), anti-caspase-3 (1:500, Cat. n° sc-7272, Santa Cruz Biotechnology, USA), anti-caspase-9 (1:750, Cat. n° 9502, Cell Signaling Technology, USA), and anti-β-Actin (1:1500, Cat. n° sc-81178, Santa Cruz Biotechnology, USA). After five washes in TBST buffer (20 mM Tris, 140 mM NaCl, 0.1% Tween-20) the membranes were incubated with appropriate secondary antibody HRP, Goat anti-Rabbit IgG (1:10.000, Cat. n° 31460, Invitrogen, Thermo Fisher® Scientific, USA) and anti-mouse IgG (1:10.000, Cat. n° 7076, Cell Signaling Technology, USA). The chemiluminescent signal was detected by the Chemidoc acquisition instrument (Bio-Rad, USA). The obtained images were analyzed with the Image Lab software (Bio-Rad, USA). If required, depending on protein molecular weight, the membranes were subjected to

stripping protocol, before proceeding with further staining. Briefly, 15′ incubation with stripping solution (Restore™ PLUS Western Blot Stripping Buffer, Cat. n° 46430, Thermo Fisher® Scientific, USA) at 37 °C.

5.1.8 Firefly luciferase assay

For validation of pro-caspase-3 as a target of miR-675-5p, HCT-116 cells were seeded at 7×10^4 cells/cm^2 and 24 hours after seeding, transfected with Attractene Transfection Reagent (Cat. n° 301005, Qiagen, Germany) for 24 hours with 100 ng (3.7 pMoles/cm^2) of mirVana™ hsa-miR-675-5p mimic (Mimic miR-675-5p, Assay ID MC12067, Thermo Fisher®, USA) or mirVana™ Scrambled Negative Control (Scr) and with 50 ng of Reporter plasmid DNA (caspase-3 Human 3′ UTR Clone/RFP, Cat. n° SC215501, OriGene Technologies, Inc) used following the manufacturer's standard application guide. Then 24 hours after transfection, luciferase tests were performed using the Firefly Luciferase Assay Kit (Cat. # PR300001, OriGene Technologies, Inc) following the manufacturer's standard instructions. Luminescence and fluorescence were detected by GloMax®-Multi Microplate Reader (Promega, USA). The luminescence was normalized for the Red Fluorescent Protein (RFP) values and the relative Luciferase activity following the overexpression of the hsa-miR-675-5p mimic (Luc/RFP + Mimic miR-675-5p) is expressed in fold change with respect to the Negative Control (Luc/RFP + Scr).

5.1.9 Statistical analysis

Data are reported as mean ± standard deviation (SD) of at least three biological replicates. Statistical analyses: Student's t-test or Ordinary one-way ANOVA with Bonferroni's multiple comparison test were performed by using GraphPad Prism software (GraphPad Software, USA). P-values were indicated in the graphs as follow: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$.

5.2 Results

5.2.1 Prolonged hypoxia induced chemoresistance to the 5-FU treatment and enhanced miR-675-5p expression

Long-time exposure to hypoxic conditions, beyond 48 hours, is known to activate molecular pathways leading cancer cells to promote survival strategies including chemoresistance [666–668]. To reproduce this condition in vitro, CRC cell lines (HCT-116 and SW480) were treated with different concentrations of 5-FU and maintained in a hypoxic chamber containing 1% O_2 gas mixture for 72 hours. The activation of hypoxic response in our model was confirmed by the increase of the carbonic anhydrase 9 (CA9), a primary HIF's target (***Figures 7A-B***) [668, 669].

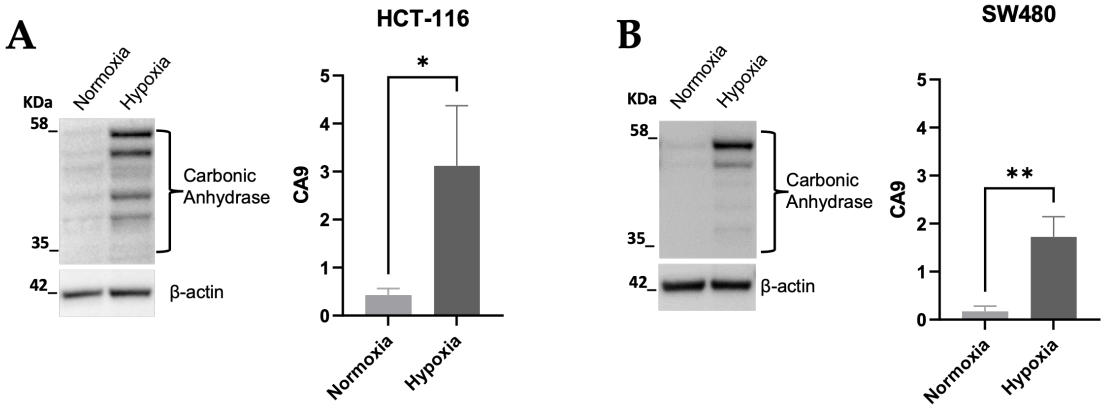


Figure 7. Carbonic Anhydrase 9 expression confirms the hypoxic condition.
A-B: Representative images and densitometric analysis of Western blots for Carbonic Anhydrase (CA9) obtained from protein lysates of HCT-116 and SW480 in normoxic conditions or subjected to hypoxic conditions. The graphs ordinate shows the OD (Optical Density) of the indicated proteins normalized for the housekeeping's OD (β-actin).
Data are expressed as the mean ± SD of three independent experiments and statistical significance was analyzed using a Student's t-test (* = $p < 0.05$; ** = $p < 0.01$).

To investigate the effects of hypoxia on cell survival, MTT assays have been done. As expected, the cell viability assay showed that 5-FU treatments induced cell death in CRC cell lines in normoxic conditions while it did not occur in hypoxic conditions (***Figures 8A-B***). These data supported the use of our model as a tool to investigate the molecular mechanisms controlling hypoxia-induced chemoresistance. Further experiments have been performed by using the higher dose of 5-FU.

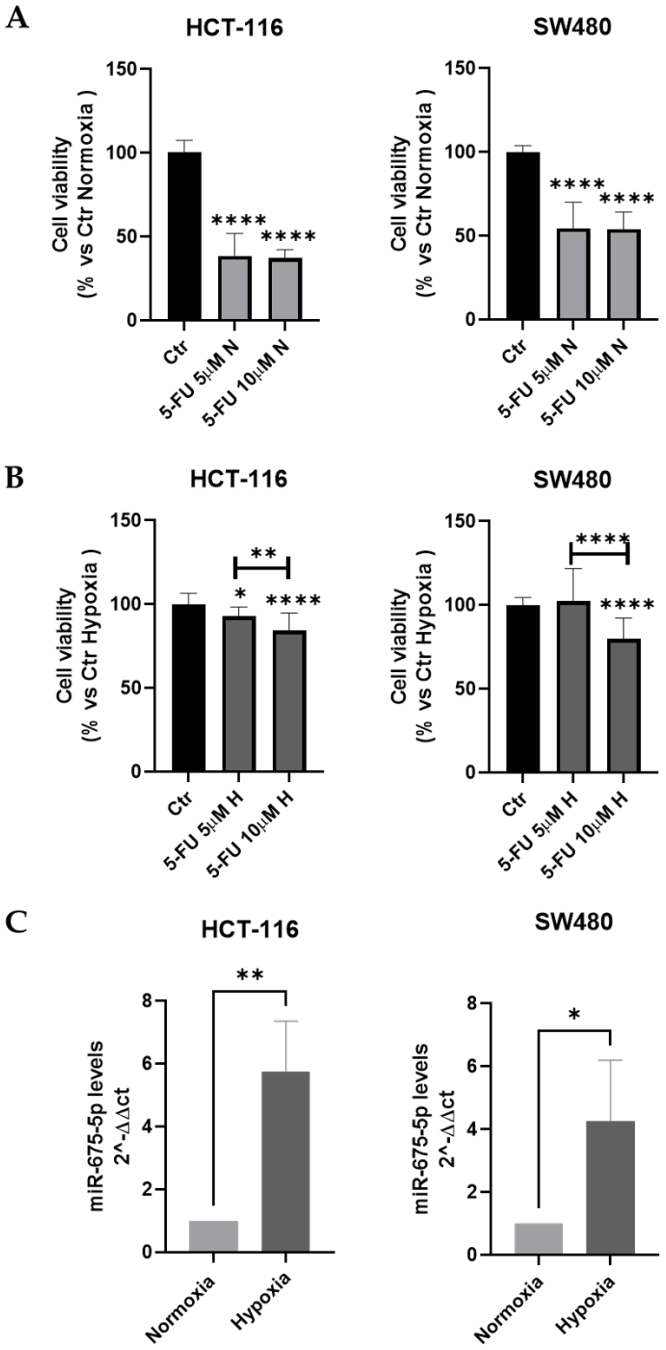


Figure 8. Colon cancer cell lines behaviour under chronic hypoxic stimulation (72 h).
A-B: Cell viability assay (MTT Assay) in HCT-116 and SW480 treated for 72 h with two different concentrations of 5-FU (5 µM and 10 µM) in normoxic (N) conditions or subjected to hypoxic (H) conditions. Data are expressed as the percentage of cell viability versus untreated cells both in normoxia and chronic hypoxia (Ctr).
C: Analysis of the expression level (qRT-PCR) of miR-675-5p in HCT-116 and SW480 under normoxic conditions and after 72 hours of hypoxic stimulation. The miR-675-5p levels were normalized for RNU6 (U6 Small Nuclear 1), and the ΔΔCt was calculated with respect to the expression levels under normoxic conditions.
All data are the mean ± SD of three biological replicates. Statistical analyses: Ordinary one-way ANOVA with Bonferroni's multiple comparison test were used in Figs. **A** and **B**, Student's t-test was used for Fig. **C** (* = $p < 0.05$; ** = $p < 0.01$; **** = $p < 0.0001$).

Our previous manuscripts identified the miR-675-5p as hypoxia-induced miRNA with a role in mediating acute hypoxic responses. However, the expression of miR-675-5p after prolonged hypoxic stimulation has not been yet investigated [480, 603]. The RT-PCR in ***Figure 8C*** revealed that CRC lines after prolonged hypoxia (72 hours) express higher levels of miR-675-5p compared to cells in normoxic conditions. These data prompted us to investigate its role in drug resistance.

5.2.2 The use of miR-675-5p antagonist counteracted the hypoxia-induced drug resistance

Firstly, through miRNA inhibition, we explored the role of hypoxia-induced miR-675-5p in cell viability. MTT assay revealed that in both cell lines, treatment with miRNA AntagomiR-675-5p reduced cell viability of hypoxic cells (***Figure 9A***). In the light of this, we investigated whether treatment with AntagomiR-675-5p could enhance the effect of 5-FU thus overcoming the hypoxia-induced chemoresistance.

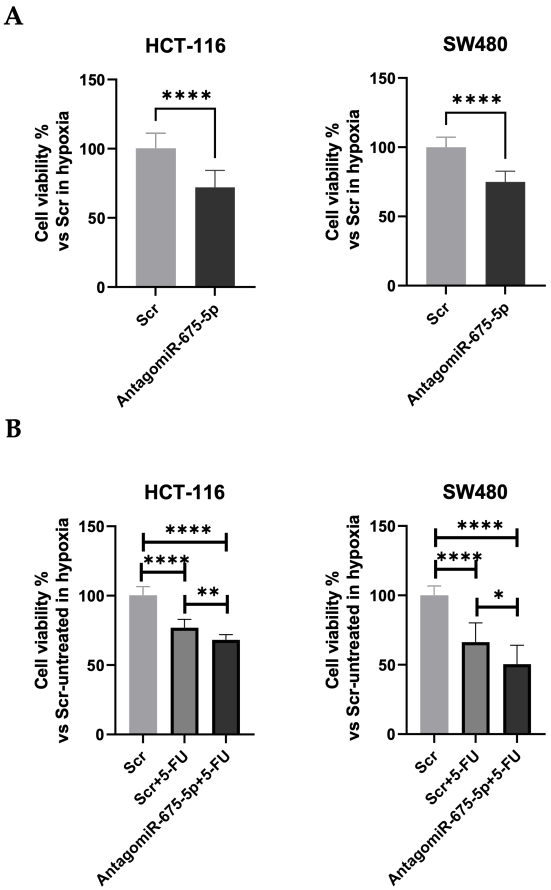


Figure 9. Effects of AntagomiR-675-5p treatment in cell viability in chronic hypoxic conditions.
A: Cell viability assay (MTT Assay) in HCT-116 and SW480 transfected with AntagomiR-675-5p or Scrambled Negative Control (Scr) and grown in the hypoxic chamber for 72 h. Data are expressed as cell viability percentage compared to cells transfected with Scr.
B: Cell viability assay (MTT Assay) in HCT-116 and SW480 transfected with AntagomiR-675-5p or Scramble Negative Control (Scr) and treated or not for 72 h of hypoxia with 5-FU (10 µM).
Data are expressed as the mean ± SD of three biological replicates. Statistical analyses: Student's t-test was used for Fig. **A**, and Ordinary one-way ANOVA with Bonferroni's multiple comparison test was used in Fig. **B** (* = $p < 0.05$; ** = $p < 0.01$; **** = $p < 0.0001$).

The cell viability assay confirmed our hypothesis indicating that, in hypoxic conditions, cells treated with both 5-FU and AntagomiR-675-5p showed a higher reduction of cell viability, compared to cells treated with the drug alone (***Figure 9B***).
It is known that 5-FU treatment in CRC promotes apoptosis through caspase-9 activation [670], here we investigated if the addition of the AntagomiR-675-5p promotes cell death by enforcing cell entrance into apoptosis. To this aim, western blot analyses for apoptotic markers were done in hypoxic cells (1% O_2 gas mixture) transfected with AntagomiR-675-5p or Scrambled Negative Control (Scr) and treated or not with 5-FU (10 µM). As shown in ***Figures 10A-B*** the treatment with 5-FU induced PARP-1 cleavage and increased the levels of cleaved caspase-3, interestingly these effects were further improved by the addition of AntagomiR-675-5p to the drug. Overall, these data indicated a role for the miR-675-5p in inhibiting apoptosis.

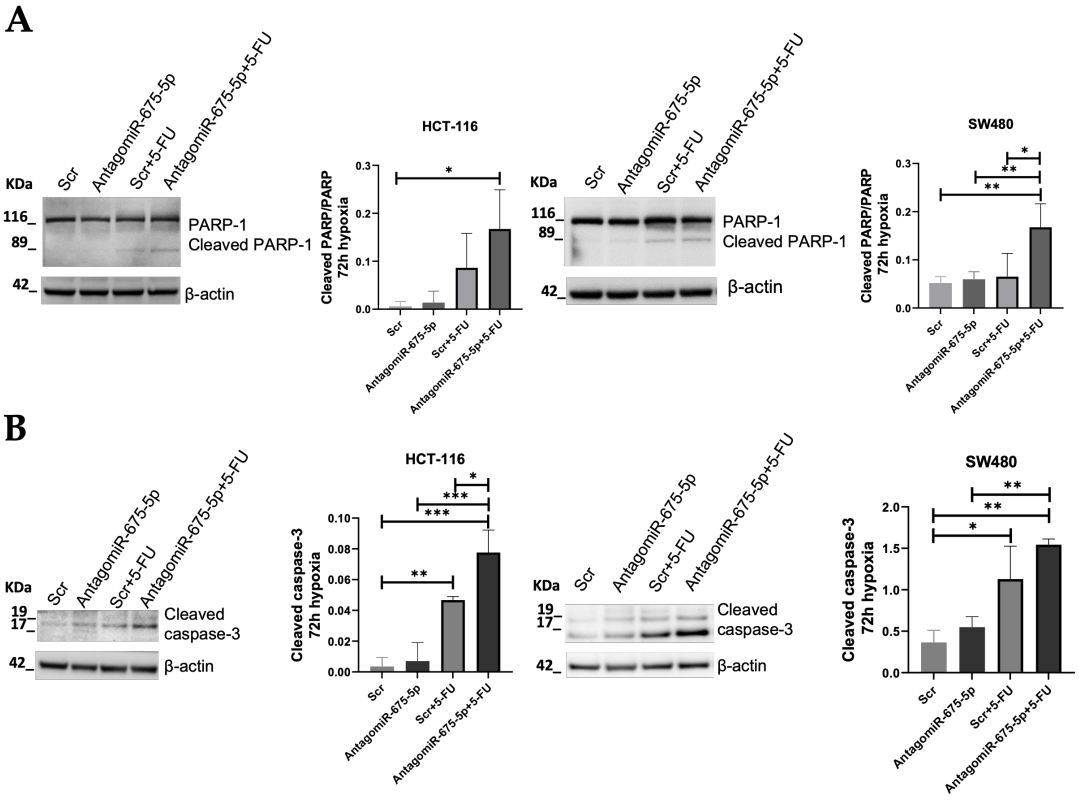


Figure 10. Effects of AntagomiR-675-5p treatment on apoptosis markers.
A-B: Representative images and densitometric analysis of Western blots for cleaved PARP-1/PARP-1 and cleaved caspase-3 obtained from protein lysates of HCT-116 and SW480 in chronic hypoxia, transfected with AntagomiR-675-5p or Scrambled Negative Control (Scr) and treated or not with 5-FU (10 µM). The graphs ordinate shows the OD (Optical Density) of the indicated proteins normalized for the housekeeping's OD (β-actin).
Data are expressed as the mean ± SD of three independent experiments and statistical significance was analyzed by using Ordinary one-way ANOVA with Bonferroni's multiple comparison test (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

5.2.3 MiR-675-5p directly targeted caspase-3 3'UTR

By querying the miRWalk database [665], we obtained the list of the putative miR-675-5p targets involved in apoptosis (***Figure 11A***) (KEGG Pathway hsa04210#Apoptosis).

Considering the effects shown by the AntagomiR-675-5p in hypoxic conditions we decided to investigate firstly the caspases of the intrinsic apoptosis pathway: caspase-9 and caspase-3. Targets' validation has been performed only in HCT-116. We transfected HCT-116 cells with miRNA-675-5p mimic (***Figure 11B***) and investigated protein levels of both putative targets. Transfection was performed on cells in normoxia as they express lower levels of miR-675-5p.

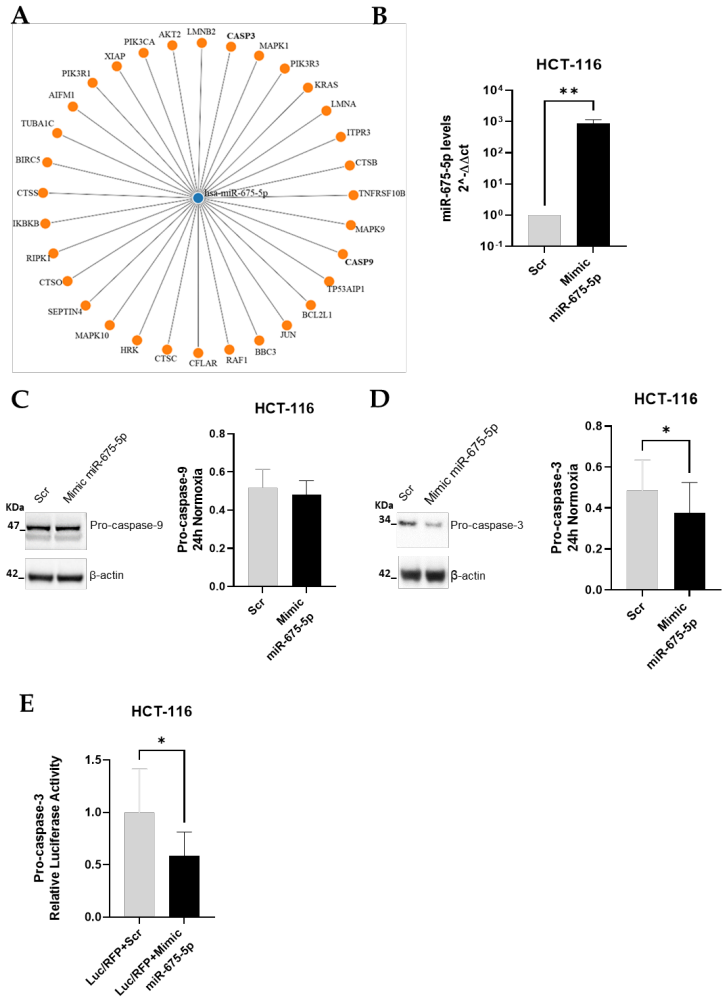


Figure 11. Identification of miR-675-5p targets involved in apoptosis.
A: The network diagram obtained using the mirWalk database [665] illustrates the presumed 3'UTR targets of miR-675-5p involved in apoptosis (KEGG Pathway hsa04210#Apoptosis).
B: Expression level analysis (qRT-PCR) of miR-675-5p in HCT-116 after overexpression of miR-675-5p under normoxic conditions. The miR-675-5p levels were normalized for RNU6 (U6 Small Nuclear 1), and the ΔΔCt was calculated with respect to the Scrambled Negative Control (Scr).
C-D: Representative images and densitometric analysis of Western blots respectively for pro-caspase-9 and pro-caspase-3 on proteins lysates from HCT-116 transfected with miR-675-5p mimic or Scrambled Negative Control (Scr) for 24 h in normoxia. The graphs ordinate shows the OD (Optical Density) of the indicated proteins normalized for the housekeeping's OD (β-actin).
E: The Firefly Luciferase assay validates pro-caspase-3 as the target of miR-675-5p. Luminescence was normalized for RFP values end presented in the graph as relative Luciferase activity in cells treated with mimic-miR-675-5p (Luc/RFP + Mimic miR-675-5p) with respect to cells treated with the Negative Control (Luc/RFP + Scr).
All Data are expressed as mean ± SD of three independent experiments and statistical significance was analyzed using Student's t-test (* = $p < 0.05$; ** = $p < 0.01$).

The western blot in ***Figure 11D*** indicated that miRNA overexpression in normoxic cells induced a reduction in pro-caspase-3 while no effects have been revealed in pro-caspase-9 (***Figure 11C***). The direct targeting of caspase-3 3'UTR, has been further confirmed by Luciferase assay (***Figure 11E***).
Overall, the data demonstrated that, in HCT-116 CRC cells grown in normoxic conditions, AntagomiR-675-5p enforces the pro-apoptotic effects of 5-FU treatment by protecting caspase-3 from miRNA-675-5p mediated inhibition.
Finally, we wondered if AntagomiR-675-5p could reinforce the effect of 5-FU even when miR-675-5p concentrations are not as high as in some cases of primary tumor or in cells under normoxic conditions [603]. ***Figures 12A-B*** indicated that, although with less intensity than in hypoxic conditions, in HCT-116 cells the use of AntagomiR-675-5p enhanced the apoptotic process induced by the 5-FU whereas AntagomiR-67-5p alone did not affect cell viability, unlike what occurred in hypoxia.

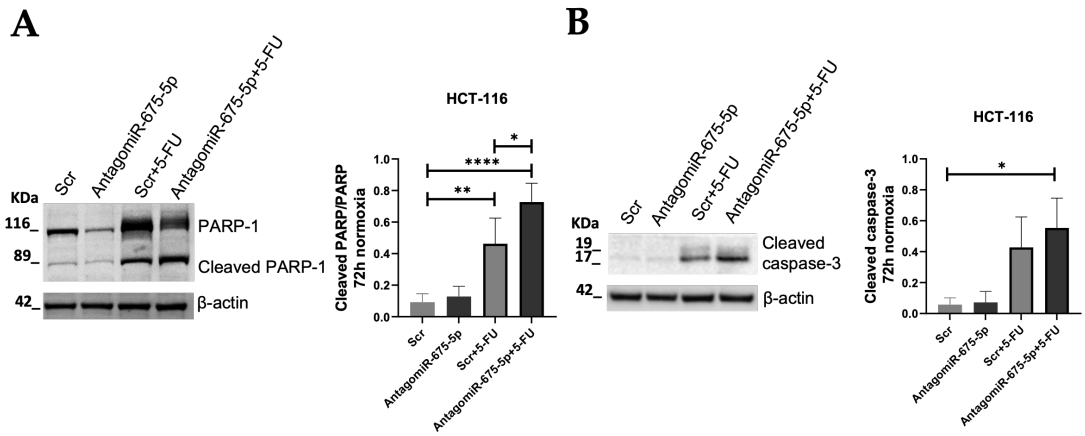


Figure 12. Effects of AntagomiR-675-5p on HCT-116 cell line in normoxic conditions.
A-B: Representative images and densitometric analyses of Western blots for cleaved PARP-1/PARP-1 and cleaved caspase-3 in HCT-116 in normoxic conditions, transfected with AntagomiR-675-5p or Scrambled Negative Control (Scr) and treated or not with 5-FU (10 μM). The graphs ordinate shows the OD (Optical Density) of the indicated proteins normalized for the housekeeping's OD (β-actin).
Data are expressed as the mean ± SD of three independent experiments and statistical significance was analysed by using Ordinary one-way ANOVA with Bonferroni's multiple comparison test (* = $p < 0.05$; ** = $p < 0.01$; **** = $p < 0.0001$).

5.3 Discussion

CRC still maintain a leading position among the causes of cancer deaths [3, 80]. Although extensive advances in CRC treatments have been reached, chemoresistance to drug treatment remains the major cause of recurrence and metastasis.
Nowadays it is important to dissect the molecular mechanisms underlying chemoresistance processes, to identify new therapeutic targets and to enhance the action of conventional therapy [113, 115, 119].
Increasing data obtained from experimental and clinical studies have shown that intratumoral hypoxia is a common feature of human cancers contributing to the development of resistance to radiation and chemotherapy [537, 671]. The response of CRC cells to treatment under low oxigen conditions aligned with previous research on the role of HIF-1 in triggering resistance to 5-FU treatment in the HCT-116 colon cancer line. These findings further validate the use of our model to investigate the molecular mechanisms that regulate hypoxia-induced resistance to chemotherapy [666, 672].
Meanwhile, several studies confirmed the role of hypoxia-induced non-coding RNAs as pivotal players mediating hypoxic responses, including drug resistance [188, 582, 583, 624, 673, 674].
Among them, we and others attributed to the lncRNA H19 and its intragenic miR675-5p an important role in promoting cancer onset and progression [258, 472, 496, 604, 675–678]. In CRC it has been demonstrated that lncH19 mediates 5-FU resistance enforcing SIRT1 mediated autophagy [679], while its expression by cancer-associated fibroblasts, promotes stemness and chemoresistance of CRC [680]. Moreover, is through the expression of its intragenic miR-675-5p that lncH19 promotes drug resistance to 1,25-dihydroxyvitamin D3 treatment; since miR-675-5p inhibits the expression of Vitamin D Receptor [676].
Thus, we assessed the levels of miR-675-5p under chronic hypoxia conditions, which as expected, given that the miRNA in question is a hypoxia-induced microRNA, was found to be significantly up-regulated compared to the normoxic condition; we therefore hypothesised its direct involvement in hypoxia-induced 5-FU chemoresistance and the possible pathways in which it might be involved. In order to understand the mechanisms underlying chemoresistance, we used loss-of-function experiments in our in vitro experimental model, aimed at specifically inhibiting the overexpressed miR-675-5p and preventing its interaction with the mRNA of the target gene, via AntagomiR-675-5p [681, 682].
Here we demonstrated, for the first time to our knowledge that the miR-675-5p, which expression is markedly increased by the hypoxic microenvironment, enforces drug resistance by affecting 5-FU induced apoptosis through the inhibition of caspase-3 (***Figure 13***).
Resistance to chemotherapy treatment is often caused by processes that inhibit the apoptosis induced by the drug, to overcome this limit several miRNAs have been identified as possible drug co-operators. MiR-206, miR-148a, miR-125a-5p and miR-129 can target BCL2, reducing its anti-apoptotic role and the overexpression of these miRNAs increased the sensitivity of CRC cells to 5-FU [683–686]. MiR-143 increased the sensitivity of colorectal cancer cells to 5-FU stimulated apoptosis by down-regulating BCL-2 and activating caspases 3, 8 and 9 [687]. Also, miR-182 by inducing caspase-3/PARP-1, and miR-34a by targeting SIRT1, significantly increase apoptosis in CRC. On the other hand, the reduction of miRNA such as miR-135b, miR-21 and miR-587, involved in apoptosis, can be considered a solution to enhance the apoptosis of CRC cells [688–690].
To verify the possible correlation between miR-675-5p and apoptosis pathways we used the miRWalk database, to obtain a network of the 3'UTR putative targets of this miRNA. We found that miR-675-5p may target many mRNAs involved in apoptosis, such as caspase-3

and caspase-9. Here we have confirmed the binding of miR-675-5p to caspase-3 however other putative markers remain to be investigated.
Moreover, our data indicated that, in prolonged hypoxia, the miR-675-5p may promote cell viability in multiple ways. MTT assay revealed that miR-675-5p inhibition reduced cell viability in hypoxic cells however, treatment with the AntagomiR-675-5p alone showed no cleavage in either caspase-3 or PARP-1. Our previous manuscript demonstrated that miR-675-5p inhibition impedes beta-catenin nuclear localization in hypoxic CRC cells inducing inhibition in Cyclin D expression. It is reasonable to assume that this inhibitory effect may be reflected in a slowing of the cell cycle. However, further data must be produced to support this hypothesis.

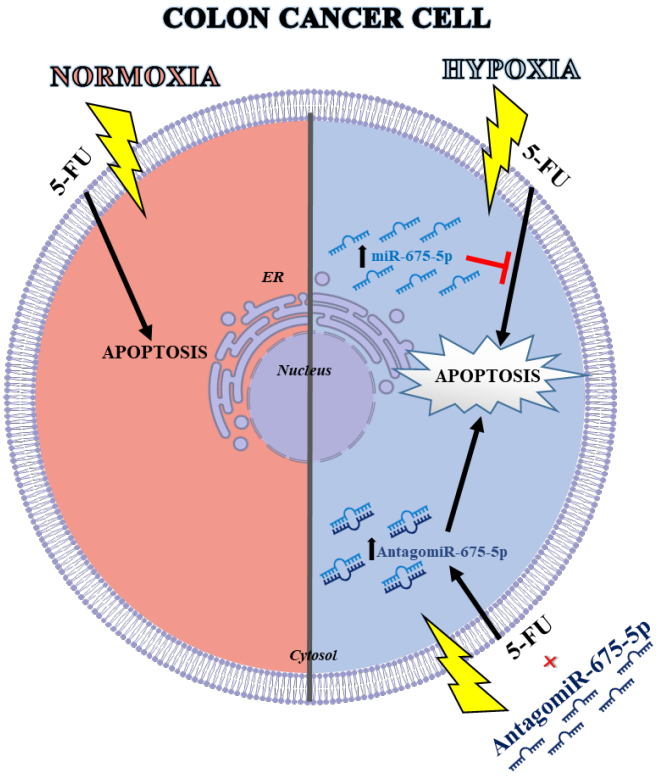


Figure 13. Schematic representation of the proposed model.
On the left in red is represented the CRC cell treated with the chemotherapeutic drug 5-Fluorouracil (5-FU) which in normoxic conditions activates the apoptotic process. In blue at the top right is represented the CRC cell treated with 5-FU in conditions of prolonged hypoxia, in which the overexpression of miR-675-5p inhibits the activation of the apoptotic process by targeting the pro-caspase-3. Finally, below on the right in blue is represented the CRC cell treated with 5-FU in conditions of prolonged hypoxia, in which the presence of AntagomiR-675-5p activates the apoptotic process, increasing the protein levels of the cleaved caspase-3 and the cleaved PARP-1.

CHAPTER 6

"LncH19 enhances the pro-apoptotic activity of ITF2357 in CRC cells"

6.1 Materials and Methods

6.1.1 Cell culture

HCT-116 cells (ATCC-LGC Standards S.r.L., Italy) were cultured in McCoy's 5A medium (Euroclone, UK) supplemented with 10% Fetal Bovine Serum, 1% Penicillin/Streptomycin (10,000 U/mL Penicillin and 10 mg/mL Streptomycin) and 200 mM L-Glutamine (all from Euroclone, UK).
5-Fluorouracil (5-FU) resistant HCT-116 cells (HCT-116-5-FU-R) were cultured in DMEM (Euroclone, UK) supplemented with 10% Fetal Bovine Serum, 1% Penicillin/Streptomycin (10,000 U/mL Penicillin and 10 mg/mL Streptomycin) and 200 mM L-Glutamine (all from Euroclone, UK) and additionally, the culture medium contained 5-Fluorouracil (5-FU, cat. n°F6627, Sigma-Aldrich, St. Louis, MO, USA) at concentrations up to 70 µM.
Cells were maintained in a humidified atmosphere of 5% CO^2 at 37 °C and used at early passages for all experiments. The culture medium was changed every 2-3 days, and cells were split at 70–80% confluence.

6.1.2 Infection with lentiviral vectors to stably silence lncH19

HCT-116 cells were stably silenced for lncH19 by lentiviral infection with H19 Human shRNA Lentiviral Particles (Cat. n° TL318197V, OriGene Technologies, Inc., Rockville, MD, USA) while relative control cells were infected with Control shRNA Lentiviral Particles (Cat. n° TR30021V, OriGene Technologies, Inc., Rockville, MD, USA). Subsequently, infected cells were selected by cell sorting (BD FACSAria™ III Sorter, ATeN Center) and maintained in culture under selective pressure with 1mg/mL of puromycin (Gibco™ Puromycin Dihydrochloride, cat. n°A1113802, Thermo Fisher® Scientific, USA). Silencing efficiency was regularly tested by qRT-PCR and fluorescence microscopy.

6.1.3 Selection of HCT-116-5-FU resistant cells

5-FU resistant HCT-116 cell line (HCT-116-5-FU-R) was established after sequential treatments with 5-FU during an 8-months period starting from 1 µM to 70 µM concentrations. Control parental cells were split in parallel. Viable cells treated with 70 µM 5-FU were considered stably resistant when the morphology resembled that of parental HCT-116.

6.1.4 Chemicals and reagents

ITF2357 (Givinostat) was synthesized and supplied by the pharmaceutical company Italfarmaco S.P.A (Cinisello Balsamo, MI, Italy). For in vitro experiments, ITF2357 was dissolved in DMSO (20 mM stock solution) and stored at -20 °C. Before use, the stock solution was thawed and diluted in McCOY'S 5A or DMEM culture media, not exceeding 0.01% (v/v) DMSO to realize the proper final concentrations.
The autophagy inhibitor Bafilomycin A1 (Cat. n° B1793-2UG, Sigma-Aldrich, USA) was solubilized in DMSO according to the data sheet instructions and used for the experiments at 20 and 50 nM final concentrations.

6.1.5 MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5 Diphenyl Tetrazolium Bromide) assay

Cell viability was determined by MTT assay following the manufacturer's instructions (Cat. n° M6494, Thermo Fisher®, USA), and the absorbance was measured by biophotometer at 540 nm (BioTek Elisa ELX800 Absorbance Microplate Reader, BioTek Instruments, USA). HCT-116 cells (wild type, silenced for lncH19 or 5-FU resistant) were seeded in at least three technical replicates at 5×10^4 cells/cm^2; 24 hours post-seeding, cells were treated with different concentrations of ITF2357 (0.25 - 0.5 - 1 - 2 or 4 µM) and maintained in a humidified atmosphere of 5% CO_2 at 37 °C. MTT assay was done after different time points as indicated in the results.
For the experiments with the autophagy inhibitor Bafilomycin A1, HCT-116 cells were pretreated for 1 hour with Bafilomycin A1 (20 and 50 nM concentrations) and then ITF2357 was added at different concentrations (0.25 - 0.5 or 1 µM) for 48 hours.

6.1.6 Colony formation assay

LncH19 silenced HCT-116 cells and control cells were seeded at 40 cells/cm^2 in six-well plates. After 48 hours, cells were treated with different concentrations of ITF2357 (0.05 - 0.1 - 0.25 and 0.5 µM) and maintained in culture for 8 days to allow clone formation. Clones were then washed once with phosphate buffer solution (PBS), fixed and stained with methylene blue 1% in PBS/ethanol 50% for 1 minute at room temperature. Following air-drying, clones were observed under a light microscope (LeicaDMR, Microsystems S.r.l, Wetzlar, Germany). Only clones containing more than 50 cells were considered and were counted. For counting, each well was divided into four quadrants and the media of the number of clones in each quadrant was estimated. The total number of clones per well was then obtained.

6.1.7 Annexin V/PI apoptosis detection assay

Annexin V/PI apoptosis detection assay (APC Annexin V Apoptosis Detection Kit with PI, cat. n° 640932, BioLegend®) was used to identify early and late apoptotic cells. LncH19 silenced HCT-116 cells and respective control cells were seeded at 1.87×10^4 per cm^2, allowed to adhere overnight and then treated with 1 µM ITF2357 for 48 hours.
Briefly, following the manufacturer's instructions, cells were harvested, and, after centrifugation, cell pellets were washed twice with cold BioLegend cell staining buffer (Cat. n° 420201), resuspended in annexin V binding buffer, and labelled with APC Annexin V and Propidium Iodide.

Approximately 50000 events were acquired for each sample on a FACSCanto cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Flow cytometry data were analyzed using FlowJo software (v10; TreeStar, Ashland, OR, USA).

6.1.8 Western blotting

H19 silenced HCT-116 cells and control HCT-116 cells were lysed using Lysis Buffer (15 mM Tris/HCl pH 7.5, 120 mM NaCl, 25 mM KCl, 1 mM EDTA, 0.5% Triton X100) supplemented with Phosphatase Inhibitor cocktail (Cat. N° 37492, Active Motif, USA) for 1.30h on ice. Cell debris was removed by centrifugation at 17,000 × g for 15 minutes at 4 °C and the supernatant, containing protein lysate, was quantified by Bradford assay method (Pierce™ Coomassie Plus Assay Kit, cat. N° 23236, Thermo Fisher Scientific, USA) using Bovine Serum Albumin (BSA, cat. n° A2153, Sigma-Aldrich, USA) as a standard. A total of 15 µg protein from each sample was separated using Bolt Bis-Tris gel 4% - 12% (Cat. n° NP0326BOX, Thermo Fisher Scientific, USA) and transferred on nitrocellulose blotting membrane (Amersham Protran Premium 0.45µm NC by GE HealthCare Life Science, UK). The membranes were stained with 0.1% Red Ponceau in 5% acetic acid to evaluate the correct loading of all samples. The membranes were incubated for 1h in blocking solution (5% milk or 5% BSA in 20 mM Tris, 140 mM NaCl, 0.1% Tween-20) and at 4 °C overnight with primary antibodies: anti-SQSTM1/p62 (1:500, cat. n° 39749S, Cell Signaling Technology, USA), anti-LC3B (1:500, cat. n° 2775S, Cell Signaling Technology, USA), anti-Poly ADP-Ribose Polymerase-1 (Anti-PARP-1, 1:500, cat. n° sc-8007, Santa Cruz Biotechnology USA), anti-cleaved caspase-3 (1:400, cat. n° 9664S, Cell Signaling Technology, USA), anti-p53 (DO-1, 1:200, cat. n° sc-126, Santa Cruz Biotechnology USA). After washing with Tris-buffered saline-Tween 20 (TBS-T, 20 mM Tris, 140 mM NaCl, 0.1% Tween-20) three times, the membrane was incubated with appropriate secondary antibody HRP, Goat anti-Rabbit IgG (1:10.000, cat. n° 31460, Invitrogen™, Thermo Fisher® Scientific, USA) and anti-mouse IgG (1:10.000, cat. n° 7076, Cell Signaling Technology, USA) at room temperature for 1h. The chemiluminescent signal was visualized by chemiluminescence solution (ECL™ Prime Western Blotting System Cytiva RPN2232) and was detected by using the Chemidoc acquisition instrument (Bio-Rad, USA). The images were analyzed using the Image Lab software (Bio-Rad, USA).
Depending on the molecular weight of the protein, if required, the membranes were subjected to stripping protocol, before proceeding with further incubation with other antibodies Briefly, 10-15 minutes incubation with stripping solution (Restore™ PLUS Western Blot Stripping Buffer, Cat. n° 46430, Thermo Fisher® Scientific, USA) at 37 °C, followed by subsequent washes in TBS-T.

6.1.9 LC3-B assay

HCT-116 cells were seeded at 5×10^4 cells/cm^2 in cell culture chamber slides (Cat. n° 94.6190.802, Sarstedt, Germany) and LC3B assay (Cat. n°L10382, LC3B Antibody Kit for Autophagy, Invitrogen™ by Thermo Fisher® Scientific, USA) was performed following the manufacturer's instructions.
Briefly, 24 hours after seeding, HCT-116 cells were treated for 24 hours with 50 µM chloroquine diphosphate (CQ, provided by the LC3B Antibody Kit for Autophagy) alone or co-treated with 50 µM chloroquine and 1 µM of ITF2357. Chloroquine blocks autophagosome-lysosome fusion, thus allowing autophagosome visualization. After treatments, cells were fixed with 4% paraformaldehyde for 15 minutes, permeabilized with 0.1% Triton X-100

for 15 minutes and incubated with diluted LC3B rabbit polyclonal primary antibody (0.5 µg/ml according to the manufacturer's instructions) for 1 hour.
DyLight™ 594 was used as secondary antibody (Goat anti-Rabbit IgG Secondary Antibody, DyLight™ 594, 1:300, cat n°35560, Invitrogen™ by Thermo Fisher Scientific, USA).
Finally, cells have been counterstained with Hoechst (Hoechst 33342, Trihydrochloride, Trihydrate, 1:1000, cat n°H3570, Molecular Probes, Life Technologies by Thermo Fisher Scientific, USA) and ActinGreen (ActinGreen™ 488 ReadyProbes™ Reagent, 1:125, cat n°R37110, Invitrogen™ by Thermo Fisher Scientific, USA). All steps have been done at room temperature. The samples were analyzed by Nikon A1 confocal microscope.

6.1.10 RNA extraction and Real-Time PCR (qRT-PCR)

Total RNA was extracted using the commercially available Macherey-Nagel™ NucleoSpin™ miRNA Kit (Cat. n°740971.250, Macherey-Nagel, Germany) according to manufacturer's instructions. The total RNA concentration was detected with the Nanodrop spectrophotometer (Thermo Fisher®, USA) and was reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription kit (Cat. n° 4368814, Applied Biosystem™, USA).
Quantitative Real-Time polymerase chain reactions (qRT-PCR) were done by using SYBR™ Green PCR Master Mix (Cat. n° 4309155, Applied Biosystems™, USA) following the manufacturer's instructions in a Step One™ Real-time PCR System Thermal Cycling Block (Applied Biosystems, Waltham, MA, USA).
The primers' sequences used for expression analysis of the genes of interest are reported in ***Table 5***. Gene expression levels were normalized using β-actin as endogenous control. Finally, data are presented as $2^{-\Delta\Delta Ct}$ compared with the untreated control.

	Forward	**Reverse**
H19	TCGTGCAGACAGGGCGACATC	CCAGCTGCCACGTCCTGTAACC
SQSTM1/p62	TGTGTAGCGTCTGCGAGGGAAA	AGTGTCCGTGTTTCACCTTCCG
MAP1LC3A	GCTACAAGGGTGAGAAGCAGCT	CTGGTTCACCAGCAGGAAGAAG
ATG16L	CTACGGAAGAGAACCAGGAGCT	CTGGTAGAGGTTCCTTTGCTGC
LAMP1	CGTGTCACGAAGGCGTTTTCAG	CTGTTCTCGTCCAGCAGACACT
LAMP2	GGCAATGATACTTGTCTGCTGGC	GTAGAGCAGTGTGAGAACGGCA
TP53	CCTGGATTGGCCAGACTGC	TTTTCAGGAAGTAGTTTCCATAGGT
NOXA	AGCTGGAAGTCGAGTGTGCT	ACGTGCACCTCCTGAGAAAA
PUMA	GGAGCAGCACCTGGAGTC	TACTGTGCGTTGAGGTCGTC
β-ACTIN	TCCCTTGCCATCCTAAAAGCCACCC	CTGGGCCATTCTCCTTAGAGAGAAG

Table 5. Primers' sequences of the genes analyzed.

6.1.11 Bioinformatic analysis

For the prediction of interactions between ncRNAs and their targets, bioinformatic analyses were performed using DIANA tools [691]. Specifically, lncH19-miRNA interactions were identified using DIANA-LncBase v.3, while miRNA-TP53 interactions were identified using DIANA-TarBase v.8.
In homo sapiens we identified 159 validated miRNAs that lncH19 directly binds and 42 validated miRNAs that directly bind the TP53 gene.
By overlaying the two datasets from DIANA-LncBase v.3 (lncH19-miRNAs interactions) and DIANA-TarBase v.8 (miRNAs-TP53 interactions) we found that lncH19 can bind 26 miRNAs that directly target the pro-apoptotic TP53 gene (***Table 6***).

miRNAs		
hsa-let-7a-5p	hsa-miR-17-5p	hsa-miR-107
hsa-let-7b-5p	hsa-miR-19a-3p	hsa-miR-125b-5p
hsa-let-7c-5p	hsa-miR-19b-3p	hsa-miR-181a-5p
hsa-let-7d-5p	hsa-miR-22-3p	hsa-miR-218-5p
hsa-let-7e-5p	hsa-miR-24-3p	hsa-miR-522-5p
hsa-let-7f-5p	hsa-miR-30a-5p	hsa-miR-940
hsa-let-7g-5p	hsa-miR-34a-5p	
hsa-let-7i-5p	hsa-miR-93-5p	
hsa-miR-10b-5p	hsa-miR-98-5p	
hsa-miR-15a-5p	hsa-miR-103a-3p	

Table 6. 26 miRNAs sponged from lncH19 that directly targeting the pro-apoptotic TP53 gene.

6.1.12 Statistical analysis

Data reported in all graphs are the mean ± standard deviation (SD) of at least three independent biological replicates. The following tests have been performed: Student's t-test to compare two groups, One-Way ANOVA for comparisons among three or more groups, and Two-Way ANOVA for comparison of multiple variables among two groups. Analyses were performed by GraphPad Prism software (GraphPad Software, USA).
P-values were indicated in the graphs as follow: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$. A p-value ≤ 0.05 was considered significant.

6.2 Results

6.2.1 ITF2357 reduces CRC cell viability and increases the expression levels of lncH19

Initially, to evaluate the sensitivity of the HCT-116 CRC cell line to ITF2357, cells were treated with different concentrations of ITF2357 for 16, 24, 48 and 72 hours. Evaluation of cell morphology indicated that the drug exerted a cytotoxic effect, which appeared after 24 hours in cells treated with 1 µM ITF2357 and was clearly evident after 48 hours either with 1 or 2 µM (***Figure 14A***). Morphological data were confirmed by MTT assay (***Figure 14B***). As expected, ITF2357 treatment reduced the viability of HCT-116 cells in a dose and time-dependent manner. About 50% reduction in viability was observed after 48 hours of treatment with 1 µM ITF2357.
LncH19 is known to display oncogenic activity in CRC, promoting cell proliferation [692], epithelial to mesenchymal transition [481], and 5-FU drug resistance [679].
To elucidate whether the HDACi modifies the expression levels of lncH19, we performed qRT-PCR analyses. Interestingly, the results revealed that ITF2357 promoted lncH19 expression in HCT-116, determining a twofold increase in the level of the lncRNA after 24 hours of treatment and almost threefold increase at 48 hours (***Figure 14C***).
Therefore, we hypothesized that lncH19 induction could be somehow functional to ITF2357 to exert its cytotoxic effect.
To verify this hypothesis, HCT-116 cells were stably silenced for lncH19, and the silencing efficiency was confirmed by gene expression analysis (***Figure 15A***). Cell viability assay in H19-silenced cells revealed that ITF2357 displayed much less efficacy under lncH19 knockdown. Indeed, the effect of ITF2357 was reduced by about 15% suggesting that lncH19 plays a role in ITF2357-induced cytotoxicity in CRC cells (***Figures 15B-C***).
Moreover, colony formation assay further confirmed a direct role of lncH19 to sustain the efficacy of the HDACi in CRC cells. Specifically, as shown in ***Figure 15D***, treatment with ITF2357 affected the clonogenicity of HCT-116 control cells in a dose-dependent manner, while this effect was significantly weaker in H19-silenced cells, as also revealed by the quantification of the number of clones in the two cell types (***Figure 15E***).

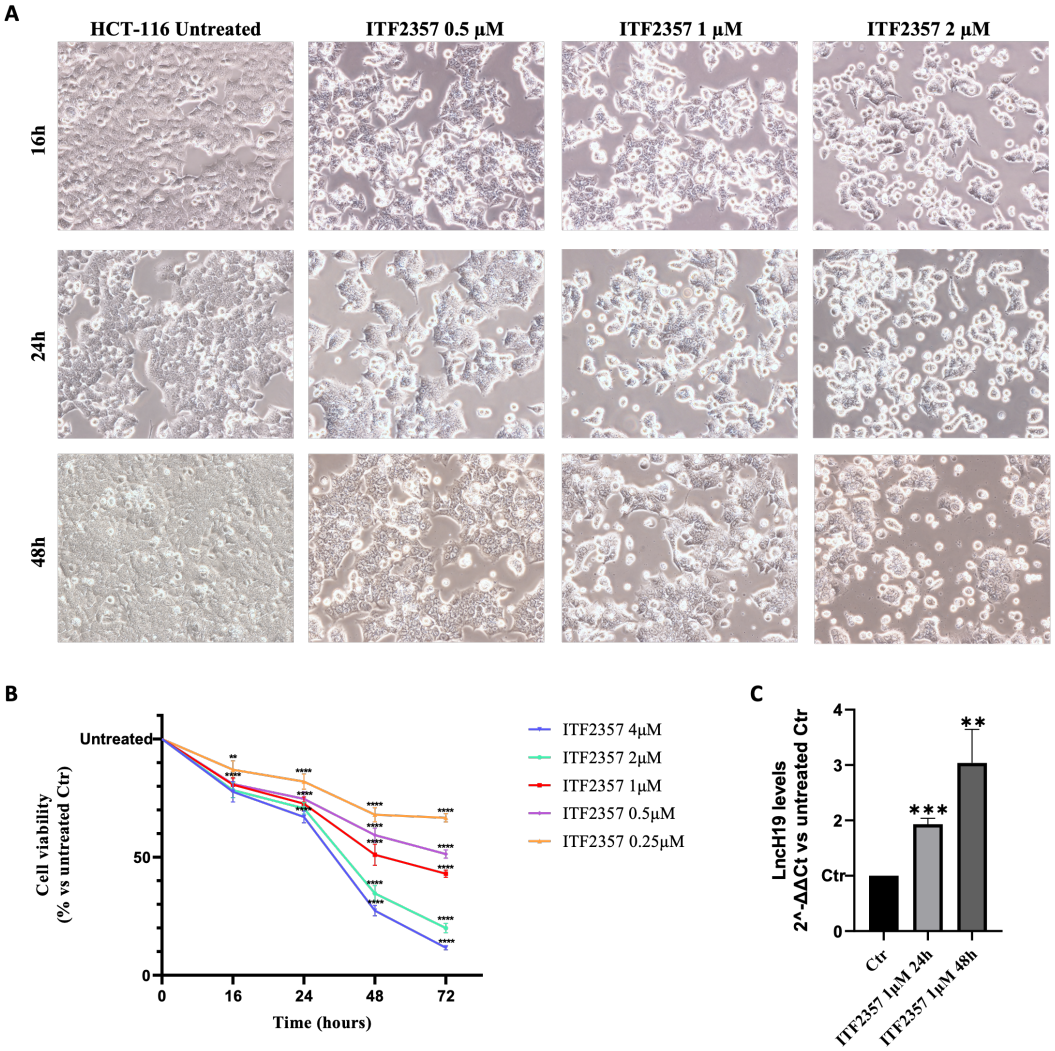


Figure 14. Effects of ITF2357 on HCT-116 cell viability and lncH19 expression.

A: Phase contrast images of HCT-116 cells treated with different concentrations of ITF2357 (0.5 - 1 and 2 µM) for 16, 24 and 48 hours. The cells were visualized under a light microscope at 20× magnification, and the pictures were acquired by NISA1 Leica Software.

B: Cell viability assay (MTT Assay) in HCT-116 cells treated with different concentrations of ITF2357 (0.25 - 0.5 - 1 - 2 and 4 µM) for 16, 24, 48 and 72 hours. Data are expressed as cell viability percentages compared to untreated cells (Ctr). The results reported in the graph are the mean ± SD of three independent biological replicates. Statistical analyses were performed using Ordinary two-way ANOVA with Bonferroni's multiple comparison test (** $p < 0.01$, **** $p < 0.0001$).

C: Analysis of the expression level (qRT-PCR) of lncH19 in HCT-116 cells treated with 1 µM ITF2357 for 24 and 48 hours. LncH19 expression level are reported as $2^{-\Delta\Delta Ct}$ compared to untreated cells (Ctr) and threshold cycle (Ct) were normalized against β-actin. The results reported in the graph are the mean ± SD of three independent biological replicates. Statistical analyses were performed using Student's t-test (** $p < 0.01$, *** $p < 0.001$).

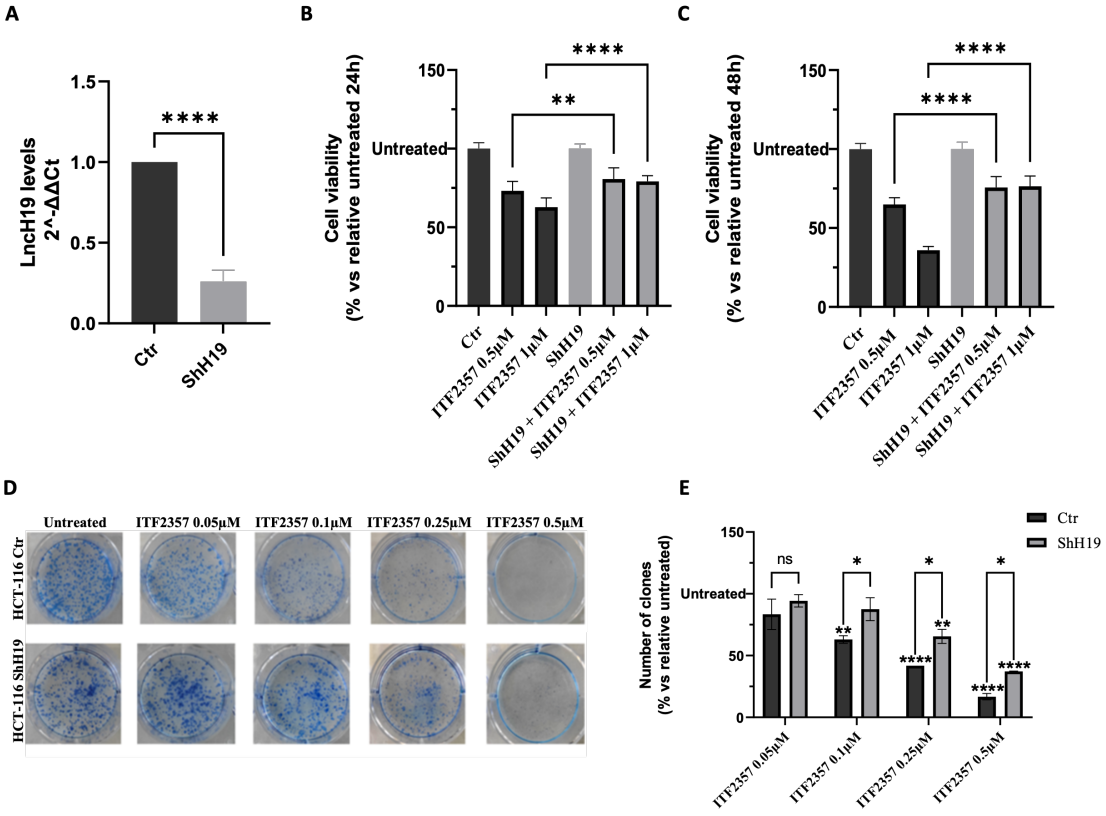


Figure 15. Effects of silencing of lncH19 in HCT-116 treated with ITF2357.
A: Analysis of the expression level (qRT-PCR) of lncH19 in HCT-116 silenced cells respect to control cells (Ctr). LncH19 expression level are reported as $2^{-\Delta\Delta Ct}$ compared to control cells (Ctr), Ct were normalized against β-actin. Data are expressed as the mean ± SD of three independent biological replicates. Statistical analyses were performed using Student's t-test (**** $p < 0.0001$).
B-C: Cell viability assay (MTT Assay) in HCT-116 cells silenced or not for lncH19 and treated with two different concentrations of ITF2357 (0.5 and 1 µM) for 24 hours (left graph) and 48 hours (right graph). Data are expressed as cell viability percentage compared to untreated cells. Data are expressed as the mean ± SD of three independent biological replicates. Statistical analyses were performed using Ordinary one-way ANOVA with Bonferroni's multiple comparison test (** $p < 0.01$, **** $p < 0.0001$).
D-E: Clonogenic assay in HCT-116 cells silenced or not for lncH19, untreated or treated with indicated concentrations of ITF2357 and maintained in culture for 8 days to allow clone formation. In the histogram data are expressed as percentage of the number of clones compared to relative untreated cells. Data are expressed as the mean ± SD. Statistical analyses were performed using Ordinary two-way ANOVA with Bonferroni's multiple comparison test (* $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$).

6.2.2 ITF2357 induces pro-survival autophagy in CRC cells

It is well known that both HDACis and lncH19 induce autophagy in different tumor cells [693–695]. Therefore, we hypothesized that ITF2357, enforced by H19 expression, induces autophagy-dependent cell death. To verify this hypothesis, the transcriptional levels of some autophagy markers (ATG16L, SQSTM1/p62, MAP1LC3B/LC3, and LAMP1/2) were analyzed. As shown in ***Figure 16A***, ITF2357 upregulated all the autophagy genes analyzed, an effect that was already evident after 24 hours. This effect was maintained after 48 hours of treatment (data not shown).
The activation of autophagy was confirmed by an increase in LC3B signal in autophagosomes, as revealed by immunofluorescence (***Figure 16B***). These data were confirmed by Western Blot analysis showing a much higher level of LC3II cleaved form in ITF2357-treated

cells. Moreover, a further confirmation of the autophagic process induced by ITF2357 was sustained by the significant decrease in the levels of p62 protein (***Figures 16C-D***). This marker is usually considered to monitor the autophagic flux and it is associated with completed autophagy when decreasing, since it is degraded by the autophagosome [696].
To investigate whether the activation of autophagy in HCT-116 cells could promote cell death, cell viability was evaluated in cells treated with ITF2357 in the presence of the autophagy inhibitor Bafilomycin A1.
As shown in ***Figure 16E***, the cytotoxic effect exerted by three different doses of ITF2357 resulted enhanced either with 20 or 50 nM Bafilomycin A1. These data suggest that autophagy induced by the HDAC inhibitor represents a pro-survival adaptive response to the effects of the compound. Moreover, we provided evidence that H19 silencing did not affect ITF2357-induced autophagy (***Figures 17A-E***).

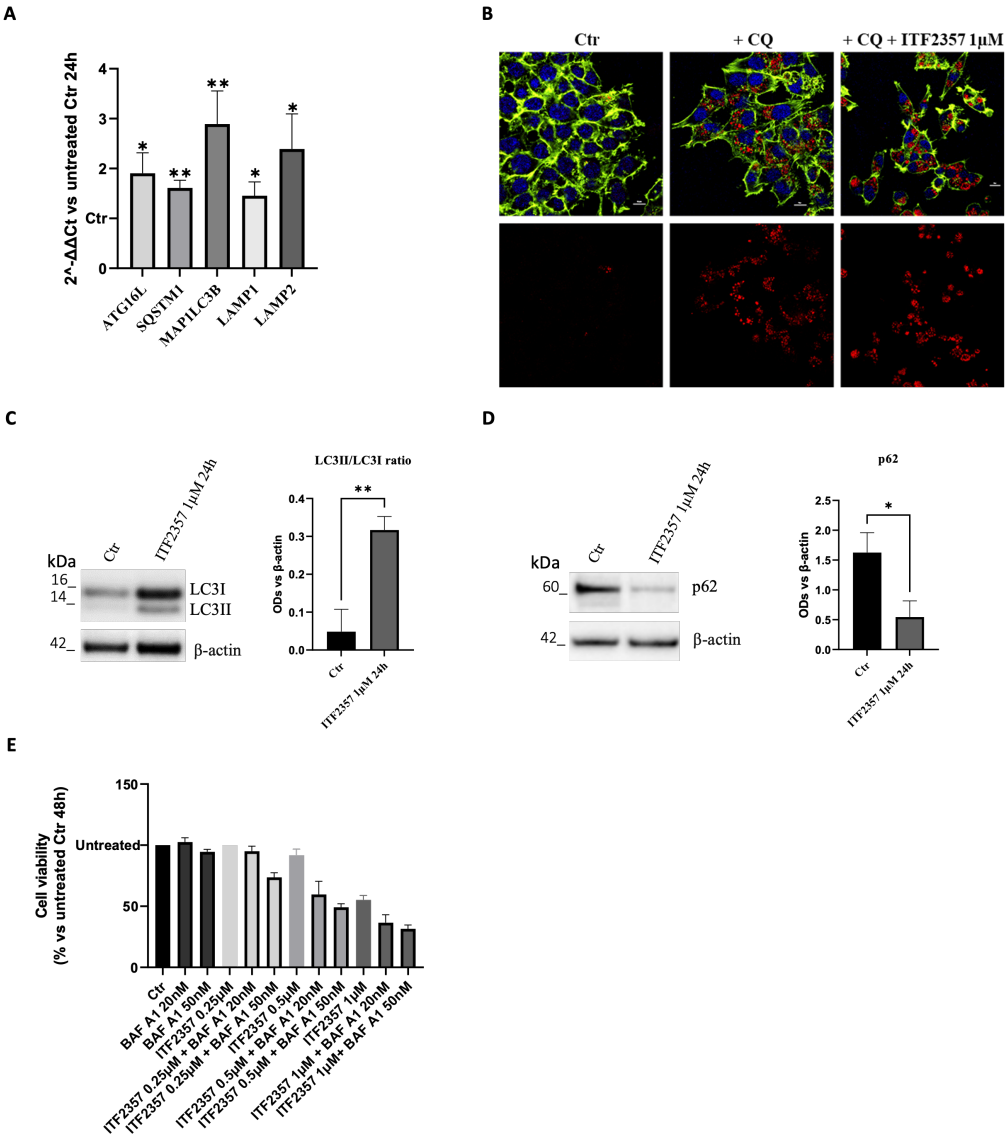


Figure 16. HDAC inhibitor ITF2357 induces survival autophagy in CRC cells.

A: Analysis of the expression level (qRT-PCR) of autophagic genes in HCT-116 cells treated with 1 μM concentration of ITF2357 for 24 hours. The expression levels of genes are reported as $2^{-\Delta\Delta Ct}$ compared to untreated cells (Ctr) and Ct were normalized against β-actin. Data are expressed as the mean ± SD of three independent biological replicates. Statistical analyses were performed using Student's t-test (* $p < 0.05$, ** $p < 0.01$).

B: Immunofluorescence for LC3B on HCT-116 cells, untreated or treated with 50 μM chloroquine diphosphate (CQ) alone or in combination with 1 μM of ITF2357 for 24 hours. LC3B in red, counterstained with Hoechst and ActinGreen, for nuclei in blue and cytoskeleton in green, respectively. Nuclear focal plane, the scale bar is 10 μm.

C: Representative images and densitometric analysis of Western Blots for LC3II/LC3I in HCT-116 cells treated or not with ITF2357 1 μM for 24 hours. The graph shows the ratio of the normalized OD (optical density). Housekeeping β-actin was used as loading control. Data are expressed as the mean ± SD of three independent biological replicates. Statistical analyses were performed using Student's t-test (** $p < 0.01$).

D: Representative images and densitometric analysis of Western Blots for p62 in cells treated or not with ITF2357 1 μM concentration for 24 hours. The graph shows the normalized OD (Optical Density). Housekeeping β-actin was used as loading control. Data are expressed as the mean ± SD of three independent biological replicates. Statistical analyses were performed using Student's t-test (* $p < 0.05$).

E: Cell viability assay (MTT Assay) in HCT-116 cells co-treated with different concentrations of ITF2357 (0.25 – 0.5 and 1 μM) and two different concentrations of Bafilomycin A1 (20 and 50 nM) for 48 hours. Data are expressed as cell viability percentages compared to untreated cells (Ctr). Data are expressed as the mean ± SD.

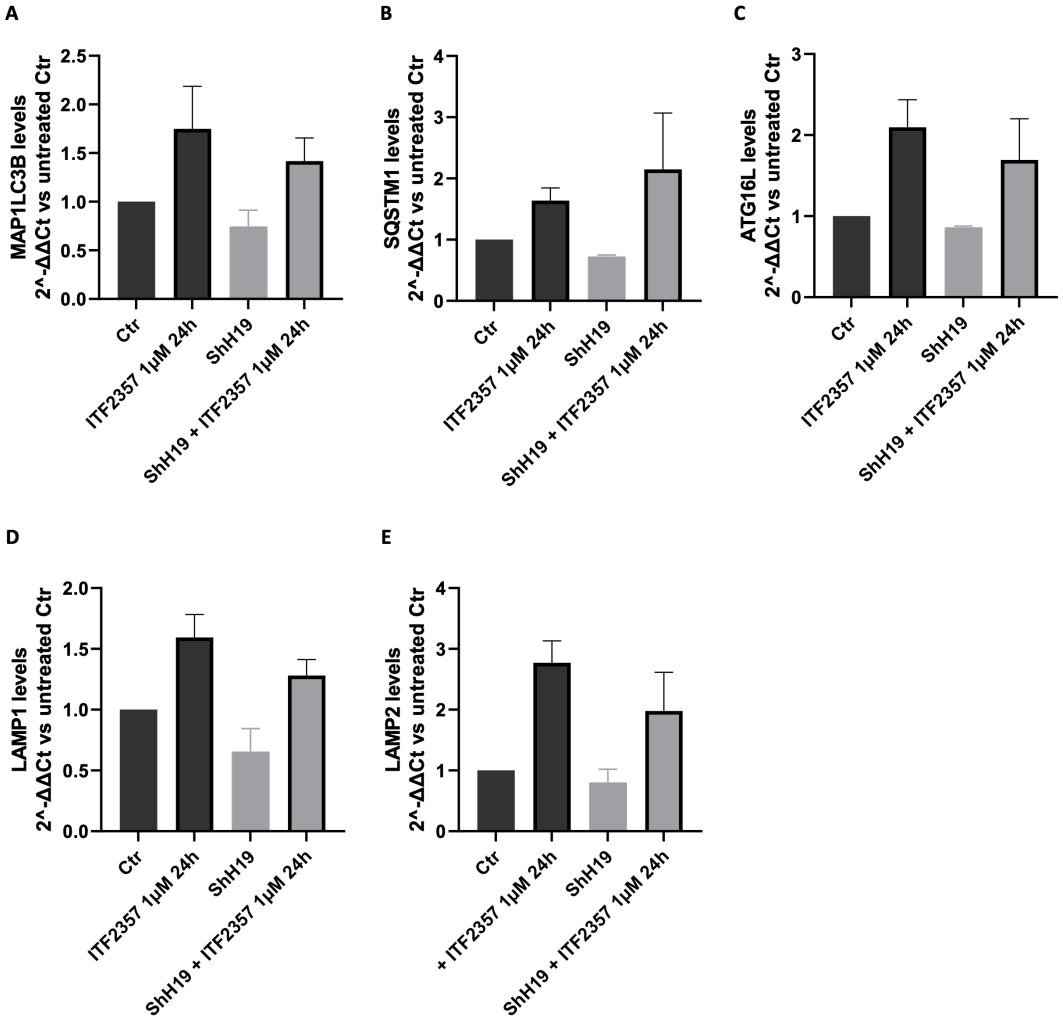
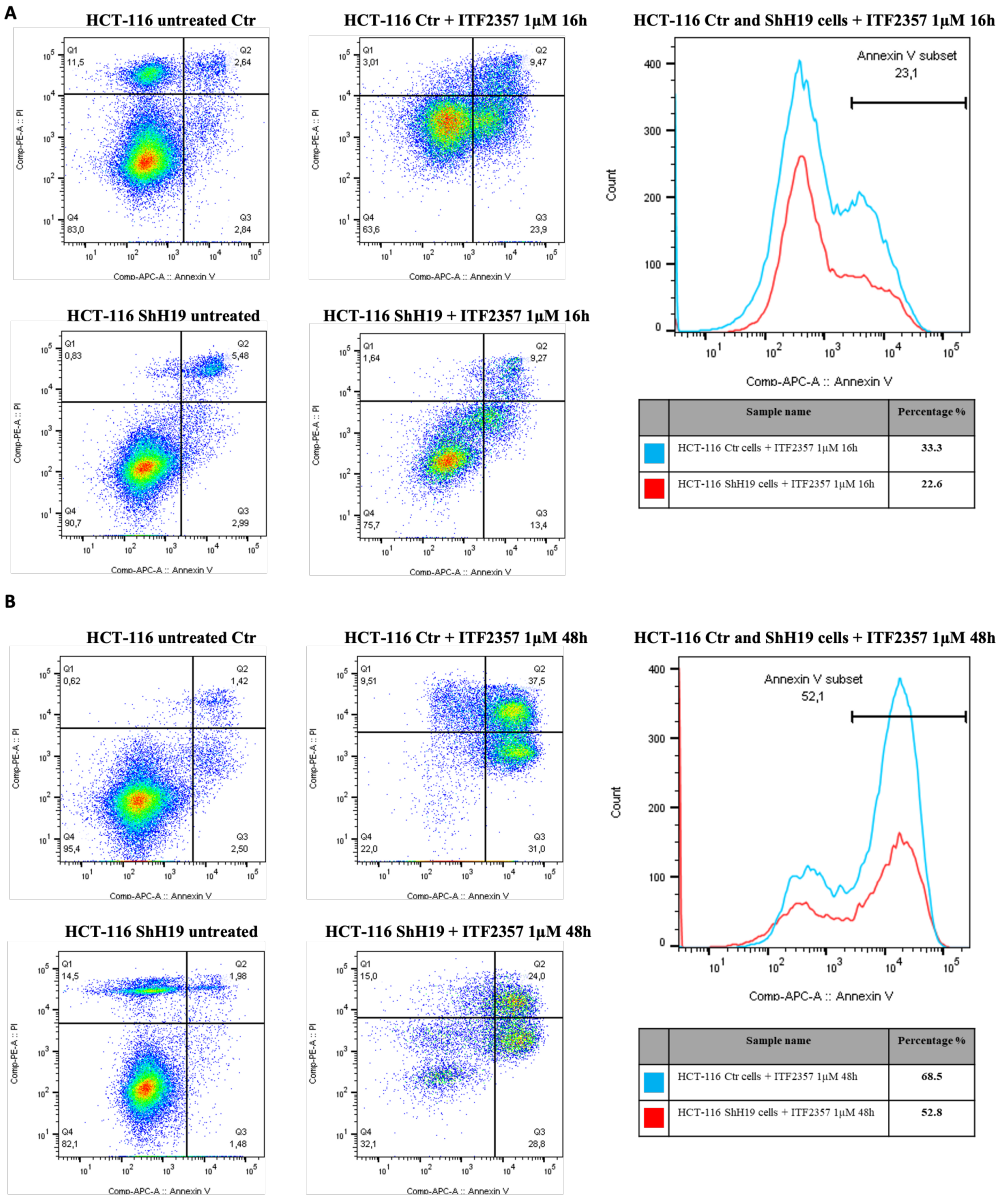


Figure 17. LncH19 silencing did not affect ITF2357-induced autophagy.
A-E: Analysis of the expression level (qRT-PCR) of autophagic genes in HCT-116 silenced for lncH19 or control cells (Ctr) untreated or treated with 1 μM concentration of ITF2357 for 24 hours. The expression levels of genes are reported as $2^{-\Delta\Delta Ct}$ compared to control cells (Ctr), threshold cycle were normalized against β-actin. Data are expressed as the mean ± SD.

6.2.3 ITF2357 induces apoptosis in HCT-116 cells and lncH19 is functional to this effect

To further characterize cell death activated in response to ITF2357 and to elucidate the role of lncH19, apoptosis was investigated in H19 silenced cells in comparison with the respective control cells. Specifically, Annexin V/PI apoptotic assay was performed at early (16 hours) and late (48 hours) treatment times points to properly detect the process along time. The results shown in (***Figures 18A-B***) indicate that ITF2357 stimulated early and late apoptosis with a different extent in control and H19-silenced cells. Indeed, the total percentage of annexin V positive cells after treatment with ITF2357 was about 33% in control cells compared to 22.6% in H19-silenced cells at 16 hours. Such a difference was maintained at 48 hours (68.6% in control cells vs 52.8% in H19-silenced cells), thus confirming that lncH19 knockdown reduces the apoptotic efficacy of ITF2357.
Morphological analysis of ITF2357-treated cells clearly showed the differential effect of the

HDACi in the two cell types (***Figure 18C***).
These data were confirmed by Western Blot analysis of apoptotic markers including cleaved caspase-3 and cleaved PARP-1, an analysis that was performed at late time points to evidence apoptosis execution. As shown in ***Figures 18D-E***, while caspase-3 cleavage and PARP-1 degradation were evident in ITF2357-treated control cells, these effects were much less evident in H19-silenced cells. These data suggest that H19 expression somehow reinforces the pro-apoptotic action of ITF2357.



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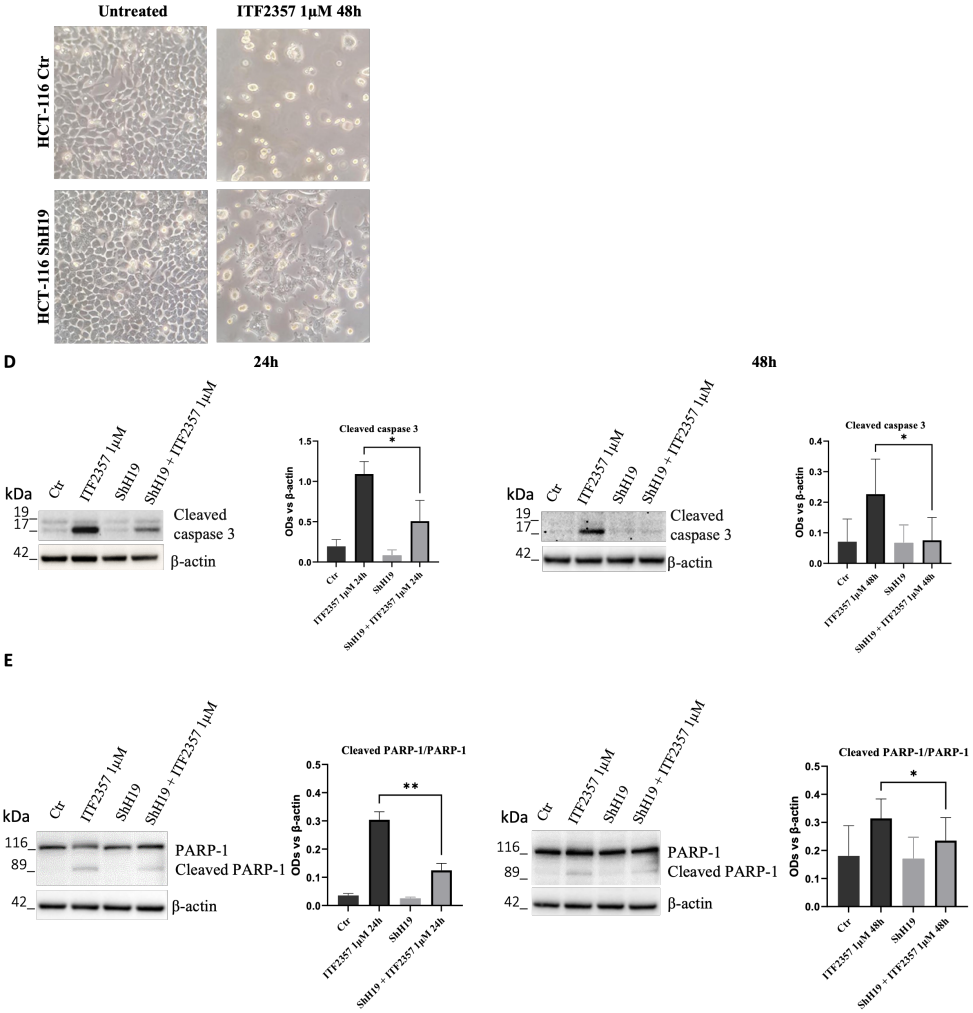

Figure 18. The effects of lncH19 silencing on apoptosis markers.
A-B: Annexin V/PI apoptosis detection assay on HCT-116 cells silenced for lncH19 or unsilenced control cells (Ctr) treated with 1 µM concentration of ITF2357 for 16 and 48 hours. Data are expressed as apoptotic cell percentage compared to untreated cells (silenced or unsilenced for lncH19).
C: Phase contrast images of HCT-116 cells silenced for lncH19 or unsilenced control cells (Ctr) untreated or treated with 1 µM of ITF2357 for 48 hours. Cells were visualized under a light microscope at 20× magnification and the pictures were acquired by IM50 Leica Software (Leica DMR Microsystems, Wetzlar, Germany).
D-E: Representative images and densitometric analysis of Western Blots for cleaved caspase-3 (**D**) and cleaved PARP-1/PARP-1 (**E**) obtained from protein lysates of HCT-116 silenced for lncH19 or control cells (Ctr) were treated with 1 µM ITF2357 for 24 or 48 hours. The graphs show the OD (Optical Density) of the indicated proteins normalized for the housekeeping's OD (β-actin). Data are expressed as the mean ± SD of three independent biological replicates. Statistical analyses were performed using Student's t-test (* $p < 0.05$, ** $p < 0.01$).

To investigate the molecular mechanism by which lncH19 promotes ITF2357-induced apoptosis we focused on identifying putative miRNAs with a pro-apoptotic role that could be targeted by lncH19. In fact, as other lncRNAs, H19 can also behave as an endogenous competitive sponge for miRNAs [462]. By using DIANA tools [691] we identified 159 validated human miRNAs sponged by lncH19 and, among these, 26 validate human

miRNAs directly targeting the pro-apoptotic TP53 gene (***Figure 19A***). Real-time PCR in ***Figure 19B*** confirmed a positive correlation between the expression of lncH19 and TP53. The transcriptional analyses revealed that cells silenced for lncH19 express lower levels of TP53 and its targets PUMA and NOXA (***Figures 19B-D***). The reduction of p53 in shH19-cells was further confirmed at the protein level (***Figure 19E***). Overall, these data indicate that ITF2357 induces TP53 mediated apoptosis in colorectal cancer cells, and the expression of lncH19 plays a functional role in regulating p53 expression.

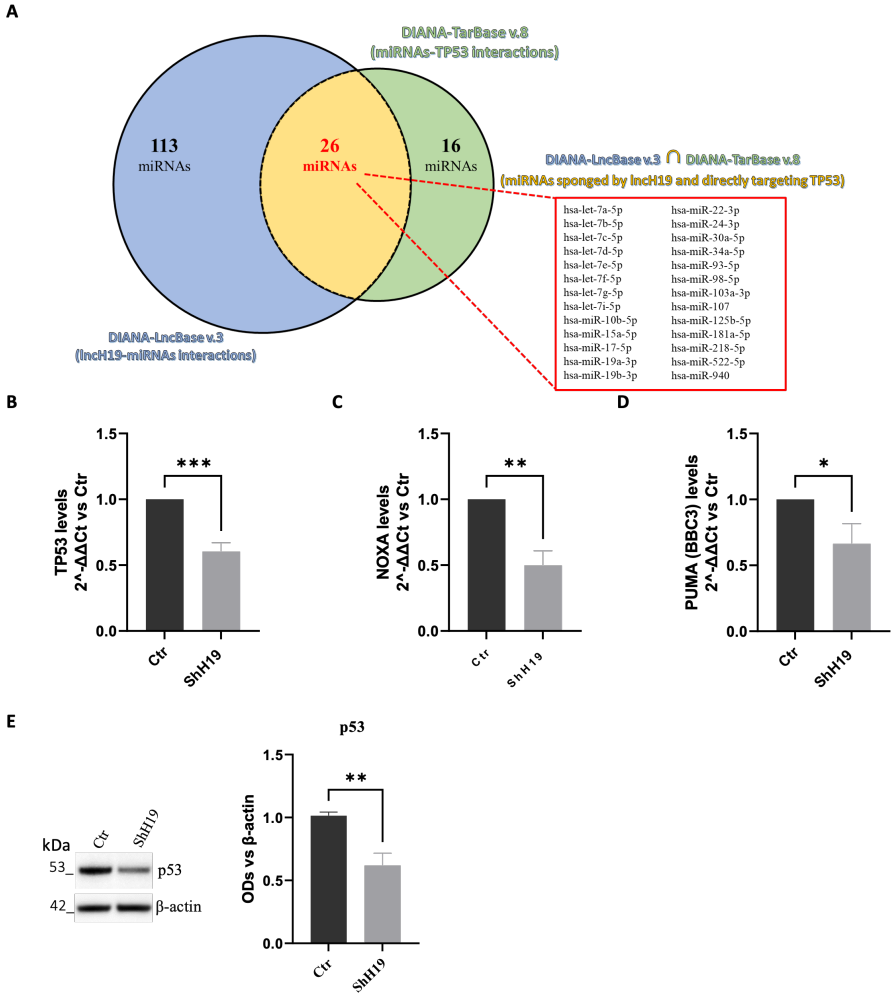

Figure 19. Identification of lncH19 miRNAs that target TP53.
A: Venn diagram obtained by bioinformatic analysis using DIANA-tools illustrating the intersection (in yellow) between the dataset of validated direct miRNAs that lncH19 binds (DIANA-LncBase v.3, in blue) and the dataset of validated miRNAs that directly bind TP53 (DIANA-TarBase v.8, in green).
The intersection shows 26 miRNAs (listed in the panel) sponged from lncH19 that directly target the pro-apoptotic TP53 gene.
B-D: Analysis of the expression level (qRT-PCR) of TP53 (**B**), NOXA (**C**) and PUMA (**D**) in HCT-116 respect to control cells (Ctr). Gene expression levels are reported as $2^{-\Delta\Delta Ct}$ compared to control cells (Ctr), Ct were normalized against β-actin. Data are expressed as the mean ± SD of three independent biological replicates. Statistical analyses were performed using Student's t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).
E: Representative images and densitometric analysis of Western Blots for p53 in HCT-116 respect to control cells (Ctr). The graph shows the OD of the indicated proteins normalized for the housekeeping's OD (β-actin). Data are expressed as the mean ± SD of three independent biological replicates. Statistical analyses were performed using Student's t-test (** $p < 0.01$).

Finally, to assess whether ITF2357 is able to overcome the resistance to 5-FU chemotherapeutic, we used HCT-116-5-FU-R, a 5-FU resistant HCT-116 cell line properly selected in our laboratory. Interestingly, HCT-116-5-FU-R cells express high levels of lncH19 compared to parental HCT-116 cells (***Figure 20A***). It is noteworthy that these cells nicely respond to ITF2357 as indicated by cell viability evaluation reported in ***Figure 20B*** that revealed a dose-dependent effect of the compound.

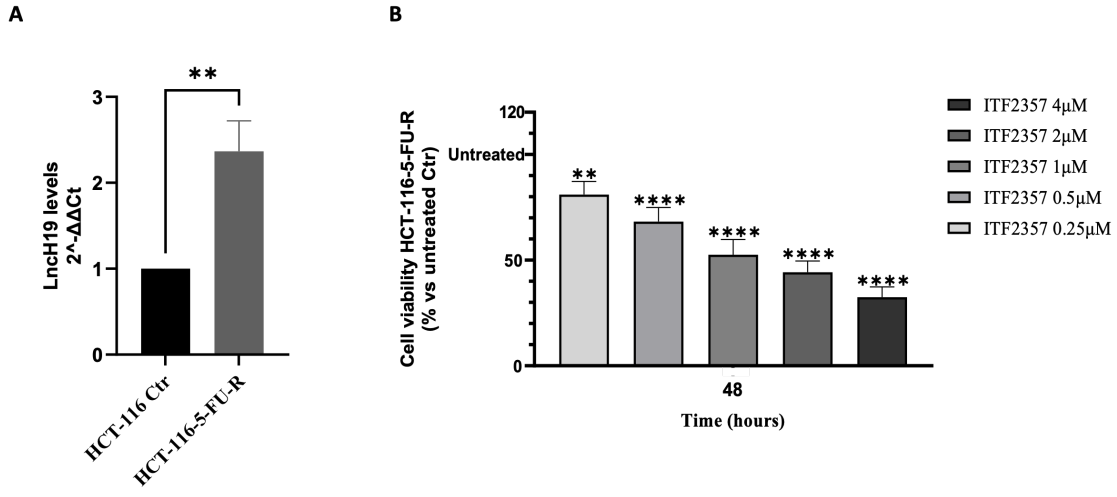

Figure 20. HCT-116 cells resistant to 5-Fluorouracil (5-FU) express high levels of lncH19 and respond to treatment with ITF2357.
A: Analysis of the expression level (qRT-PCR) of lncH19 in HCT-116-5-FU-R cells compared to untreated cells (HCT-116 Ctr). LncH19 expression level are reported as $2^{-\Delta\Delta Ct}$ compared to HCT-116 Ctr cells and were Ct normalized against β-actin. The results reported in the graph are the mean ± SD of three independent biological replicates. Statistical analyses were performed using Student's t-test (** $p < 0.01$).
B: Cell viability assay (MTT Assay) in HCT-116-5-FU-R cells treated with different concentrations of ITF2357 (0.25 - 0.5 - 1 - 2 and 4 µM) for 48 hours. Data are expressed as cell viability percentages compared to untreated cells (Ctr). The results reported in the graph are the mean ± SD of three independent biological replicates. Statistical analyses were performed using Ordinary one-way ANOVA with Bonferroni's multiple comparison test (** $p < 0.01$, **** $p < 0.0001$).

6.3 Discussion

The presented data show, for the first time, that lncH19 supports apoptosis induced by HDACi ITF2357 in colon cancer cells. Although some papers sustain the potential of HDACis in colon cancer treatment [150, 697], to date, no evidence has been provided about the efficacy of this pan-HDACi in colon cancer cells. Our data indicate that ITF2357 is active in colon cancer cells at micromolar concentrations, in line with the findings of other authors in different tumor cell lines [142, 145, 148, 698]. We also provided evidence that ITF2357 upregulates lncH19 in colon cancer cells. Similarly, Di Fazio et al. found increased lncH19 levels in adrenocortical carcinoma, following treatment with pan-HDACis such as panobinostat, trichostatin A (TSA), and SAHA, correlated with autophagy induction [699]. To understand the role of lncH19 in ITF2357-induced cytotoxicity in colon cancer cells, both autophagy and apoptosis induction were examined in H19 stably silenced HCT-116 cells in comparison with control HCT-116 cells. It is well known that HDACis can promote autophagy in different tumor types [700–702]. However, it is well known that autophagy can exert a dual role in tumor cells. Indeed, the process can be activated as a pro-survival response, which is frequently associated with tumor progression and chemoresistance, or it can serve a death-inducing function, thereby representing an alternative form of cell death to target tumor cells that have developed apoptosis resistance [129]. This work shows that ITF2357 promoted the expression of autophagy markers, including ATG16L, SQSTM1/p62, MAP1LC3B/LC3, and LAMP1/2. HDACi also induced the conversion of LC3I into active LC3II and a reduction in the levels of p62. Our data support the hypothesis that ITF2357-induced autophagy is correlated with a pro-survival cell response since the autophagy inhibitor bafilomycin A1 markedly potentiated the cytotoxic effect of the compound and the p62 protein marker decreased, indicating autophagy completion [696]. Our findings are in accordance with the observation of Angeletti et al., who found that inhibition of autophagy potentiates the effect of ITF2357 in glioblastoma cells [698]. However, our data ***Figures 17A-E*** indicates that lncH19 silencing does not significantly modify the levels of autophagy markers.

Therefore, we concluded that the cytotoxic effect of ITF2357 does not depend on autophagy-induced cell death, and subsequently, caspase-dependent apoptosis was considered.

Evaluation of apoptosis by annexin V/PI double staining and analysis of apoptotic markers revealed that lncH19 plays a role in this event. Indeed, ITF2357-induced apoptosis was reduced in H19-silenced cells compared to the respective control cells. We consider these results relevant since they imply that lncH19 can be exploited to favor apoptosis induction and that HDACi may promote a H19-dependent targeted effect in colon cancer cells. In accordance with our results, other authors have previously found a correlation between lncH19 and apoptosis.

In particular, Hou et al. have shown that overexpressed lncH19 alleviates induced lung injury in mice, as well as lipopolysaccharide (LPS)-induced apoptosis, oxidative stress, and inflammation [703]. Similarly, Yang provided evidence that H19 silencing alleviates LPS-induced apoptosis and inflammation by regulating the miR-140-5p/TLR4 axis in cell models of pneumonia [704]. In a more specific tumoral context, lncH19 has been shown to participate in triptolide/TNF-α-induced apoptosis via binding miR-204-5p in gastric cancer models [705]. In addition, Liu et al. demonstrated that lncH19 inhibits proliferation and enhances apoptosis of nephroblastoma cells by regulating the miR-675/TGFBI axis [706]. Accordingly, lncH19 has also been implicated in sensitization to X-ray and carbon ion irradiation of non-small cell lung cancer [707], and positively modulates the sensitivity of glioma cells to radiation-favoring apoptosis [708]. However, some controversial data

are present in the literature regarding the pro-apoptotic role of lncH19. For instance, the knockdown of H19 in resveratrol-treated cancer cells has been shown to enhance the effects of resveratrol on apoptosis [709]. Other evidence of an antiapoptotic role of lncH19 was provided by Wang et al., who showed that it promotes proliferation, migration, and invasion, and inhibits apoptosis of breast cancer cells by targeting the miR-491-5p/ZNF703 axis [710]. It is clear that lncRNA H19 and many other cellular factors may exert a dual role in regulating cell fate [711].

Our data strongly suggest a pro-apoptotic role of lncH19 in CRC cells treated with HDACi ITF2357 since lncH19 silencing profoundly reduced the effects of the compound on cell viability and apoptosis. To explain the pro-apoptotic role of lncH19 in HDACi-treated cells, we hypothesized that it may act as an endogenous competitive sponge for miRNAs [459], antagonizing miRNAs targeting pro-apoptotic genes. Bioinformatic analysis revealed that lncH19 sponged 26 validated human miRNAs directly targeting the pro-apoptotic gene TP53 (***Figure 21***).

Our data provide evidence that lncH19 knockdown reduces the expression of TP53 and its pro-apoptotic targets, PUMA and NOXA. The relationship between lncH19 and TP53 is controversial in the literature since some papers sustain a negative control of TP53 by H19 [487, 712, 713], while others support that lncH19 may activate the tumor suppressor. Specifically, in accordance with our findings, we have shown that overexpression of lncH19 enhanced TP53 expression, whereas H19 silencing exerted the opposite effect [714]. In addition, Du et al. have found that lncH19 promotes p53 phosphorylation by a direct interaction, an effect that results in increased NOTCH-mediated angiogenesis in mesenchymal stem cells [715].

Interestingly, our data also provided evidence that lncH19 is overexpressed in HCT-116-5-FU-R cells, and we consider it relevant that the HDACi ITF2357 was capable of overcoming 5-FU resistance in these cells. Other authors have associated 5-FU resistance with lncH19 expression [679, 716, 717]; here, we suggest that this condition may be exploited to promote TP53-dependent apoptosis using HDACi. To date, several lines of evidence indicate that HDACi can sensitize different tumor types to the effects of diverse chemotherapeutic agents [718–722].

Overall, our data suggest that lncH19 levels may be a useful parameter to promote epigenetic targeting of colon cancer and propose ITF2357 as a promising epi-drug in colon cancer treatment.

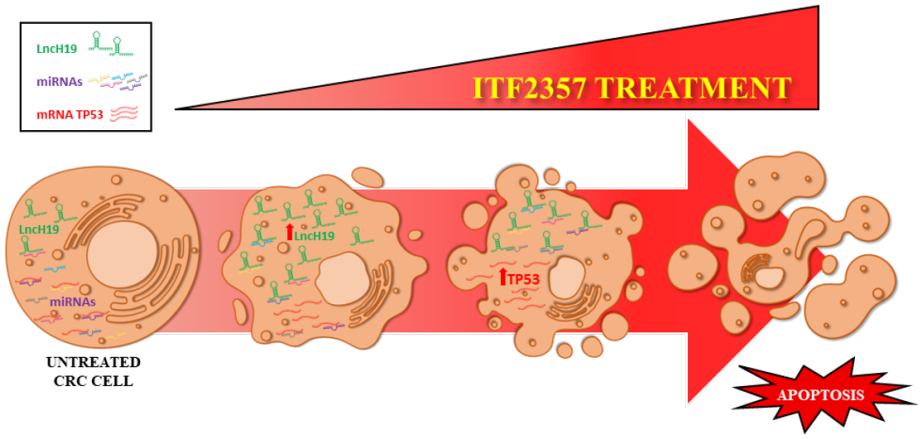

Figure 21. Schematic representation of the proposed model.
The levels of lncH19 increase in CRC cells treated with HDACi ITF2357. This increases the sponge effect by lncH19 on miRNAs targeting pro-apoptotic genes, including TP53. Overall, treatment with ITF2357 increases lncH19 levels and promotes activation of apoptosis, thus leading to increased expression of TP53.

CHAPTER 7

Conclusions

Recent evidence suggests that non-coding RNAs (ncRNAs) play a significant functional role in cancer progression and drug resistance. This highlights the possible use of ncRNAs for clinical and translational applications in cancer treatment. [723–725].
This doctoral project aimed to investigate the role of lncH19 and its intragenic miRNA, miR-675, in controlling tumor progression and chemoresistance in CRC cells, with the ultimate goal of identifying novel targets and therapeutic strategies to enhance conventional therapy.
The literature shows that both of these ncRNAs are up-regulated in CRC and regulate multiple key aspects of tumorigenesis, including cell proliferation, apoptosis, metastasis formation and drug resistance mechanisms [188, 499, 603, 679].
For the first time to our knowledge, our data revealed a dual role of the lncRNA H19 and its miRNA, as both therapeutic targets and as putative prognostic biomarkers. Indeed, our data demonstrated that lncH19 enforces CRC cell resistance to 5-FU, especially under chronic hypoxic conditions, through its intragenic miRNA; on the other hand, its expression seems to be functional for the anti-tumor activity of the epi-drug as for the HDACi ITF2357.

The first part of the project was dedicated to study the response of colorectal cancer cells to 5-FU treatment under chronic hypoxic conditions and to verify the expression and possible role of the ncRNAs of our interest in this experimental model.
Loss-of-function experiments, bioinformatic analyses and luciferase assays have allowed us to demonstrate that miR-675-5p functions as an "onco-miR", promoting the establishment of drug resistance processes by inhibiting the apoptotic process by down-regulating caspase-3 (***Figure 13***). The results obtained identified miR-675-5p as a possible therapeutic target for CRC patients, further revealing the positive use of AntagomiR-675-5p as an adjunct to drug treatment; in fact, this could lead to a reduction in therapeutic drug doses, thus reducing dose-dependent related effects.
Indeed, its downregulation enhances 5-FU activity under both hypoxic and normal conditions, thus revealing a possible strategy to overcome, at least in part, drug resistance in the hypoxic tumor microenvironment.

A further effort in overcome 5-FU drug resistance is done by the use of histone deacetylase inhibitors (HDACis). Recently, clinical trials have shown that these compounds act by sensitizing tumors resistant to conventional chemotherapy [726]. Therefore, the second part of the project focused on studying the use of a specific HDACi, ITF2357, for the treatment of lncH19 expression-positive CRC.
Specifically, we found that ITF2357 induces cell death in HCT-116 colon cancer cells upregulating H19 expression.
Although many data in the literature describe H19 as a lncRNA with canonically oncogenic,

pro-apoptotic and chemoresistance-promoting functions [679, 727, 728], our data showed a different aspect of it.
Our experiments demonstrated that lncH19, is functional to the pro-apoptotic activity of the ITF2357, in fact, under HDACi treatment, the lncRNA acts as a ceRNA for miRNAs, by stabilizing TP53 (***Figure 21***).
In addition, we provided evidence for the first time that HDACi ITF2357 is valuable as chemotherapy in a colon cancer model, by upregulating lncH19 and overcoming 5-FU resistance in highly H19-expressing CRC cells.

In conclusion, it is necessary to consider that the results obtained with the present PhD project refer to CRC cell lines, with all the limitations of in vitro studies.
Although in vivo studies are needed to confirm our data, our studies reinforce the evidence that ncRNAs can be used as therapeutic adjuvants to enhance the response to drug treatments.

CHAPTER 8

Scientific Products

8.1 List of publications or other scientific products produced within the project and relevant to the topic

8.1.1 Scientific publications in journals

Scientific Review: ***Hypoxia-Induced Non-Coding RNAs Controlling Cell Viability in Cancer.***
Authors: Maria Magdalena Barreca[1], **Chiara Zichittella**[1], Riccardo Alessandro[1,2], Alice Conigliaro[1].
[1] *Department of Biomedicine, Neurosciences and Advanced Diagnostics (Bi.N.D.), Section of Biology and Genetics, University of Palermo, 90133 Palermo, Italy.*
[2] *Institute for Biomedical Research and Innovation (IRIB), National Research Council (CNR), 90146 Palermo, Italy.*
International Journal of Molecular Sciences 2021 Febrary 12; 22(4):1857.
DOI: 10.3390/ijms22041857. PMID: 33673376.

Scientific Article: *Mir-675-5p supports hypoxia-induced drug resistance in colorectal cancer cells*.*
Authors: **Chiara Zichittella**[1], Maria Magdalena Barreca[1,2], Aurora Cordaro[1], Chiara Corrado[1], Riccardo Alessandro[1,3], Alice Conigliaro[1].
[1] *Department of Biomedicine, Neurosciences and Advanced Diagnostics (Bi.N.D.), Section of Biology and Genetics, University of Palermo, 90133 Palermo, Italy.*
[2] *Department of Biological, Chemical and Pharmaceutical Sciences and Technologies (STEBICEF), University of Palermo, 90128, Palermo, Italy.*
[3] *Institute for Biomedical Research and Innovation (IRIB), National Research Council (CNR), 90146 Palermo, Italy.*
BMC Cancer. 2022 May 20; 22(1):567.
DOI: 10.1186/s12885-022-09666-2. PMID: 35596172.
**Winner of Best Paper Award 2023 - FIRST PLACE (preclinical area), Di.Chir.On.S., University of Palermo, 2023 December 11, Palermo (Italy).

Scientific Article: ***Long non-coding RNA H19 enhances the pro-apoptotic activity of ITF2357 (Histone Deacetylase Inhibitor) in colorectal cancer cells.***
Authors: **Chiara Zichittella**[1], Marco Loria[1], Adriana Celesia[2], Diana Di Liberto[2], Chiara Corrado[1], Riccardo Alessandro[1,3], Sonia Emanuele[2], Alice Conigliaro[1].
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[2] *Department of Biological, Chemical and Pharmaceutical Sciences and Technologies (STEBICEF), University of Palermo, 90128, Palermo, Italy.*
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Frontiers in Pharmacology. 2023 September 28; 14:1275833.
DOI: 10.3389/fphar.2023.1275833. PMID: 37841928.

8.1.2 Abstracts and posters presented at scientific congresses

Scientific Videoposter: ***Hypoxia induced miR-675-5p controls cell survival by modulating apoptosis and autophagy.***
Authors: **Chiara Zichittella**[1]*, Maria Magdalena Barreca[1], Chiara Corrado[1], Aurora Cordaro[1], Riccardo Alessandro[1], Sonia Emanuele[1], Alice Conigliaro[1].
[1] *Department of Biomedicine, Neurosciences and Advanced Diagnostics (Bi.N.D.), Section of Biology and Genetics, University of Palermo, 90133 Palermo, Italy.*
Scientific videoposter presented by Chiara Zichittella at the 93th SIBS-1925 (Italian Society of Experimental Biology) Congress.
**Winner of the Best Videoposter Award presented at the 93th SIBS-1925 Congress, Stress Session, 2021 April 22-25, Palermo (Italy).

Scientific Videoposter: ***Long non-coding RNA H19 enhances the pro-apoptotic activity of histone deacetylase inhibitor ITF2357 in colorectal cancer cells.***
Authors: **Chiara Zichittella**[1]*, Marco Loria[1], Adriana Celesia[1], Chiara Corrado[1], Riccardo Alessandro[1,2], Sonia Emanuele[1], Alice Conigliaro[1].
[1] *Department of Biomedicine, Neurosciences and Advanced Diagnostics (Bi.N.D.), Section of Biology and Genetics, University of Palermo, 90133 Palermo, Italy.*
[2] *Institute for Biomedical Research and Innovation (IRIB), National Research Council (CNR), 90146 Palermo, Italy.*
Scientific videoposter presented by Chiara Zichittella at the 95th SIBS-1925 (Italian Society of Experimental Biology) Congress.
**Winner of the Best Videoposter Award presented at the 95th SIBS-1925 Congress, Experimental Oncology Session, 2023 April 12-15, Trieste (Italy) and published in Journal of Biological Research 2023.

Scientific Oral Communication: ***Long non-coding RNA H19 enhances the pro-apoptotic activity of histone deacetylase inhibitor ITF2357 in colorectal cancer cells.***
Authors: **Chiara Zichittella**[1], Marco Loria[1], Chiara Corrado[1], Riccardo Alessandro[1,2], Sonia Emanuele[1], Alice Conigliaro[1].
[1] *Department of Biomedicine, Neurosciences and Advanced Diagnostics (Bi.N.D.), Section of Biology and Genetics, University of Palermo, 90133 Palermo, Italy.*
[2] *Institute for Biomedical Research and Innovation (IRIB), National Research Council (CNR), 90146 Palermo, Italy.*
Flash oral communication presented by Chiara Zichittella at the 7th Cancer World Congress on 2023 May 29-31, Palermo, Italy.

8.2 List of publications or products (carried out by the PhDst in collaboration within the time frame of the project) not related to the project

8.2.1 Scientific publications in journals

Scientific Review: ***Molecular Mediators of RNA Loading into Extracellular Vesicles.***
Authors: Chiara Corrado[1], Maria Magdalena Barreca[1,2], **Chiara Zichittella**[1], Riccardo Alessandro[1,3], Alice Conigliaro[1].
[1] *Department of Biomedicine, Neurosciences and Advanced Diagnostics (Bi.N.D.), Section of Biology and Genetics, University of Palermo, 90133 Palermo, Italy.*
[2] *Department of Biological, Chemical and Pharmaceutical Sciences and Technologies (STEBICEF), University of Palermo, 90128 Palermo, Italy.*
[3] *Institute for Biomedical Research and Innovation (IRIB), National Research Council (CNR), 90146 Palermo, Italy.*
Cells. 2021 November 30; 10(12):3355.
DOI: 10.3390/cells10123355. PMID: 34943863.

8.2.2 Abstracts and posters presented at scientific congresses

Scientific Poster: ***Effect of the Colorectal cancer cell-derived exosomal lncRNA H19 on Human umbilical vein endothelial cells: new hypothesis, on the promotion of endothelial to mesenchymal transition.***
Authors: **Chiara Zichittella**[1], Marco Loria[1], Maria Magdalena Barreca[1,2], Aurora Cordaro[1], Simona Fontana[1], Riccardo Alessandro[1,3], Alice Conigliaro[1].
[1] *Department of Biomedicine, Neurosciences and Advanced Diagnostics (Bi.N.D.), Section of Biology and Genetics, University of Palermo, Italy.*
[2] *Department of Biological, Chemical and Pharmaceutical Sciences and Technologies (STEBICEF), University of Palermo, 90128 Palermo, Italy.*
[3] *Institute for Biomedical Research and Innovation (IRIB), National Research Council (CNR), 90146 Palermo, Italy.*
Scientific poster presented by Chiara Zichittella in the "Venice Winter School" - Extracellular Vesicles: from biophysical to translational challenges, on 2023 February 06-10, Venice (Italy).

Scientific Abstract: ***The long non-coding RNA regulates RBFOX2-mediated alternative splicing in colorectal cancer.***
Authors: Maria Magdalena Barreca[1], Aurora Cordaro[1], Marco Loria[1], **Chiara Zichittella**[1], Claudia Moltalto[2], Marco Tripodi[2], Simona Fontana[1], Riccardo Alessandro[1], Alice Conigliaro[1].
[1] *Department of Biomedicine, Neurosciences and Advanced Diagnostics (Bi.N.D.), Section of Biology and Genetics, University of Palermo, 90133, Palermo, Italy.*
[2] *I.R.C.C.S.Lazzaro Spallanzani, Italy.*
Oral communication presented by Maria Magdalena Barreca in International Conference on Cancer and Oncology Research (online), 2023 June 19-20, Roma (Italy).

Scientific Poster: ***Horizontal transfer of long non-coding RNA H19 transports splicing factors in recipient cells.***

Authors: Marco Loria[1], **Chiara Zichittella**[1], Maria Magdalena Barreca[1], Aurora Cordaro[1], Simona Fontana[1], Riccardo Alessandro[1], Alice Conigliaro[1].
[1] *Department of Biomedicine, Neuroscience and Advanced Diagnostics (Bi.N.D.), Section of Biology and Genetics, University of Palermo, 90133, Palermo, Italy.*
Biennial Congress of ABCD (Association of Cell Biology and Differentiation), 2023 September 21-23, Paestum (Italy).

Scientific Poster: ***The long non-coding RNA H19 regulates alternative splicing in colorectal cancer.***
Authors: Aurora Cordaro[1], Maria Magdalena Barreca[1], Marco Loria[1], **Chiara Zichittella**[1], Simona Fontana[1], Riccardo Alessandro[1], Alice Conigliaro[1].
[1] *Department of Biomedicine, Neuroscience and Advanced Diagnostics (Bi.N.D.), Section of Biology and Genetics, University of Palermo, 90133, Palermo, Italy.*
Biennial Congress of ABCD (Association of Cell Biology and Differentiation), 2023 September 21-23, Paestum (Italy).

Scientific Abstract: ***The long non-coding RNA H19 regulates RBFOX2-mediated alternative splicing in colorectal cancer.***
Authors: Maria Magdalena Barreca[1], Aurora Cordaro[1], Marco Loria[1], **Chiara Zichittella**[1], Claudia Coronello[2], Simona Fontana[1], Riccardo Alessandro[1], Alice Conigliaro[1].
[1] *Department of Biomedicine, Neuroscience and Advanced Diagnostics (Bi.N.D.), Section of Biology and Genetics, University of Palermo, 90133, Palermo, Italy.*
[2] *Fondazione Ri.MED, Italy.*
Oral communication presented by Maria Magdalena Barreca in 1st International Cancer Science Congress 2023, 2023 October 5-7, Palermo (Italy).

Scientific Poster: ***Intragenic miRNAs: molecular mechanisms for miR-675 maturation from long non-coding H19.***
Authors: Aurora Cordaro[1], Maria Magdalena Barreca[1], Marco Loria[1], **Chiara Zichittella**[1], Riccardo Alessandro[1], Alice Conigliaro[1].
[1] *Department of Biomedicine, Neuroscience and Advanced Diagnostics (Bi.N.D.), Section of Biology and Genetics, University of Palermo, 90133, Palermo, Italy.*
European Molecular Biology Laboratory Symposium, 2023 October 11-14, Heidelberg (Germany).

Scientific Poster: ***Unveiling new mechanisms of action for the lncH19 in promoting colorectal cancer.***
Authors: Maria Magdalena Barreca[3], Aurora Cordaro[3], **Chiara Zichittella**[3], Marco Loria[3], Claudia Montaldo[2], Claudia Coronello[1], Riccardo Alessandro[3], Alice Conigliaro[3].
[1] *Fondazione Ri.MED, Italy.*
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[3] *Department of Biomedicine, Neuroscience and Advanced Diagnostics (Bi.N.D.), Section of Biology and Genetics, University of Palermo, 90133, Palermo, Italy.*
Scientific poster presented by Alice Conigliaro in European Molecular Biology Laboratory Symposium, 2023 October 11-14, Heidelberg (Germany).

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