Universitat Autònoma de Barcelona

EV-associated miRNAs as a new source of biomarkers in endometrial, colorectal and lung cancers

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EV-associated miRNAs as a new source of biomarkers in endometrial, colorectal and lung cancers

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El ritme del cos, la melodia de la ment i l'harmonia de l'ànima
creen la simfonia de la vida.

B.K.S. Iyengar

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ABBREVIATIONS

3'-UTR	three prime untranslated region
ACS	American Cancer Society
AFM	Atomic force microscopy
AICR	American Institute for Cancer Research
AJCC	American Joint Cancer Committee
BT	Brachytherapy
CA125	Cancer antigen 125
CI	Confidence interval
CRC	Colorectal cancer
CRUK	Cancer Research UK
CSF	cerebrospinal fluid
CT	Computed tomography
CTC	Computed tomography colonography
CTCs	Circulating tumor cells
ctDNA	circulating tumor DNA
CXR	Chest radiograph
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
EBUS	Endobronchial ultrasound
EC	Endometrial cancer
ECM	Extracellular matrix
EEC	Endometrioid endometrial cancer
ELISA	Enzyme-linked immunosorbent assay
ESGE	European Society for Gastrointestinal Endoscopy
ESGO	European Society of Gyneacologic Oncology
ESMO	European Society of Medical Oncology

ESTRO	European Society of Radiotherapy and Oncology
EU	European Union
EUS	Endoscopic ultrasound
EVs	Extracellular vesicles
FACS	Fluorescence-activated cell sorting
FIGO	International Federation of Gynecology and Obstetrics
FIT	Faecal immunochemical test
g	Gram
G	Grade
gFOBT	Guaiac faecal occult blood test
HPV	Human Papillomavirus
LC	Lung cancer
LDCT	Low-dose computed tomography
LVSI	Lymphovascular space invasion
M	Metastasis
miRNA	Micro RNA
mL	milliliter
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MVBs	Multivesicular bodies
MVs	Microvesicles
N	Nodes
ND	Not determined
NEEC	Non-endometrioid endometrial cancer
NLST	National Lung Cancer Screening Trial
nm	nanometers
NPF	Negative predictive factor
NSCLC	Non-small cell carcinoma
NTA	Nanoparticle tracking analysis

PET	Positron emission tomography
RALS	Robot assisted laparoscopic surgery
RNA	Ribonucleic acid
RT	Radiotherapy
SCLC	Small cell carcinoma
SEM	Scanning electron microscopy
SEMS	Self-expandable metal stents
T	Tumor
TEM	Transmission electron microscopy
TVS	Transvaginal ultrasonography
UICC	Union for International Cancer Control
USA	United States of America
WCRF	World Cancer Research Fund and the American Institute for Cancer Research

INTRODUCTION

1. ENDOMETRIAL CANCER

1.1 Epidemiology

Endometrial cancer (EC), a tumor originating in the endometrium, is the most common gynecologic malignancy of the female genital tract and the sixth most common cancer in women worldwide. With an estimated 570,000 cases and 311,000 deaths in 2018 worldwide, this disease ranks as the fourth most frequently diagnosed cancer in women, representing the 6.6% of new cases, and the fourth leading cause of cancer female death, representing the 7.5% of deaths (Figure 1) [1].

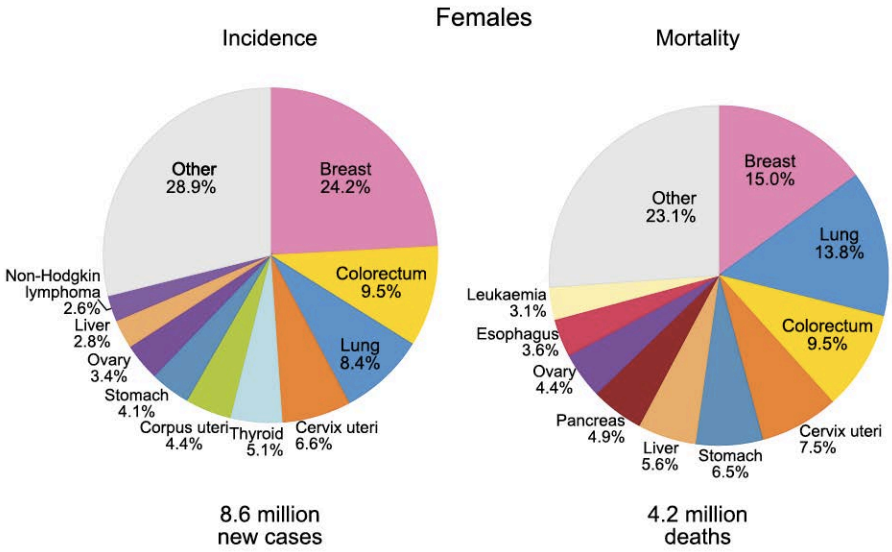


Figure 1. New cases and deaths for cancer. Pie charts representing the distribution of cases and deaths for the 10 most common cancers in 2018 for females. Adapted from Bray et al [1].

The elevated incidence rates of EC do not translate into high mortality rates thank to the early presentation of early disease-related symptoms. This permits to early diagnose most of EC patients (67%) when the tumor is still confined to the uterus and the 5-year survival rate is 95%. Unfortunately, there are still 30% of patients diagnosed at advanced stages of the disease that present a bad prognosis and a

drastic decrease in the 5-years survival rate, which goes down to 69% in cases of regional metastasis, and even lower (16%) if the metastasis is distant (Figure 2). Interestingly, cancer survival has improved since the mid-1970s for most cancers types except for few cancers, such as EC [2].

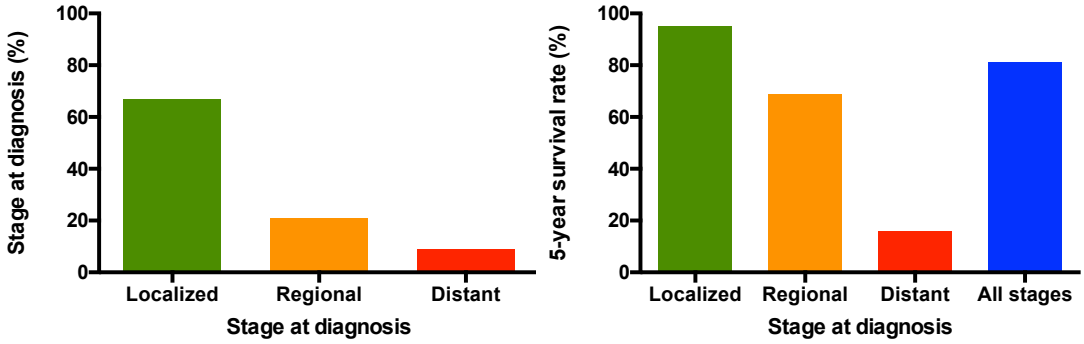


Figure 2. Stage distribution and 5-year relative survival rates of EC by stage at diagnosis. Adapted from Siegel et al [2].

1.2 Risk and Protective factors

Although the etiology of EC remains unclear, there are several risk factors proven to be associated with its development. Up to date we know that about 5% of ECs are caused by hereditary susceptibility. The main inherited factors associated with an increased risk of developing EC are the lynch syndrome and the BRCA mutation. Moreover, age is a clear risk factor associated to EC. Almost 90% of ECs cases are diagnosed in postmenopausal women, and only 14% of cases are diagnosed in premenopausal women, 5% of whom are younger than 40 years. Other most important risk factor for EC is excessive/unbalanced estrogen exposure. This excess of estrogen exposure can be due to exogenous (i.e. tamoxifen therapy for breast cancer treatment) or endogenous factors (i.e. early age at menarche, late menopause, nulliparity, infertility and chronic anovulation). Obesity, diabetes, high dietary fat intake, older age (≥55 years), metabolic disorders (lipid and carbohydrate catabolism) and hypertension could be other risk factors to take in account [3].

Some preventive factors to develop EC have also been identified. For example, there is increasing evidence that the use of combined oral contraceptives pregnancy decreases the risk of endometrial neoplasia, reducing its incidence in pre-menopausal and perimenopausal women because of the increasing of the progesterone action in these situations [4].

1.3 Diagnosis

1.3.1 Signs and symptoms

EC is usually diagnosed at early stages because 90% of the patients presents abnormal vaginal bleeding, the most common symptom of EC [5]. However, other benign disorders (Figure 3) generate this symptom and consequently, the probability of a woman with abnormal uterine bleeding having EC is only 10–15%. Although abnormal vaginal bleeding is the most frequent symptom of EC, other symptoms can be also observed such as abdominal or pelvic pain, abdominal distension, thin white or clear vaginal discharge in postmenopausal women, alterations in bowel or bladder functions, anemia and shortness of breath. Nevertheless, these are less frequent and/or associated with more advanced stages of the disease [3, 6]. However, any woman with suspicion of EC due to abnormal uterine bleeding and/or any other symptom related to EC, particularly if they have risk factors for this disease, needs to undergo a through diagnostic evaluation [7].

Causes of abnormal vaginal bleeding

- ✓ EC (15%)
- ✓ intake of exogenous estrogens (30%)
- ✓ atrophic endometritis and vaginitis (30%)
- ✓ presence of polyps (10%)
- ✓ endometrial hyperplasia (5%)
- ✓ others (10%)

Figure 3. Causes of abnormal vaginal bleeding.

1.3.2 Screening

The American Cancer Society (ACS) recommends that all women older than 65 years should be informed of the risks and symptoms of EC and advised to seek evaluation if symptoms occur. There is no indication that population-based screening has an impact in the early detection of EC among women with an average EC risk and without symptoms. Also, there is no evidence that screening by ultrasonography reduces mortality in these cases. Although being a risk factor, there are no recommendations on screening for EC in patients who are taking tamoxifen; however, women at increased risk for EC due to a history of unopposed estrogen therapy, women with late menopause, tamoxifen therapy, nulliparity, infertility or failure to ovulate, obesity, diabetes or hypertension should be properly informed of the risks and symptoms of EC and strongly encouraged to report any unexpected bleeding or spotting to their gynecologist [8]. Screening of EC is only recommended on women who have or are at increased risk of Lynch syndrome. Those women should be screened annually starting at age 35.

1.3.3 Diagnostic procedure

Clinical examination

The first steps in the current diagnostic process include a pelvic examination and a transvaginal ultrasonography to measure the thickness of the endometrium.

- Pelvic examination

  There are few physical examination findings in women with EC. A pelvic examination is used to evaluate the vulva for irritations, lesions or abnormal vaginal discharge, and then also the internal organs to evaluate if they are enlarged or tender. The uterus and adnexa should be palpated for unusual masses. Abnormal physical examination findings with respect to the size, shape and consistency of the uterus may be suggestive of more advanced disease [8] (Figure 4A).

- Transvaginal ultrasonography (TVS)

  TVS is the diagnostic imaging technique that is often the initial diagnostic study of choice when evaluating for EC because of its availability, cost-effectiveness, its simplicity and non-invasiveness. TVS can be used to measure endometrial thickness and it is a technique very effective for symptomatic patients to discard the presence of polyps and other benign pathologies. When thickening of endometrial line is detected (> 5 mm) this technique has a sensitivity and specificity of 90% and 54%, respectively [9]. Despite TVS presents a high sensitivity, the low specificity is a handicap, as other benign conditions increase the endometrial thickness and, hence, a definitive diagnosis usually requires the pathological examination of an endometrial biopsy (Figure 4B).

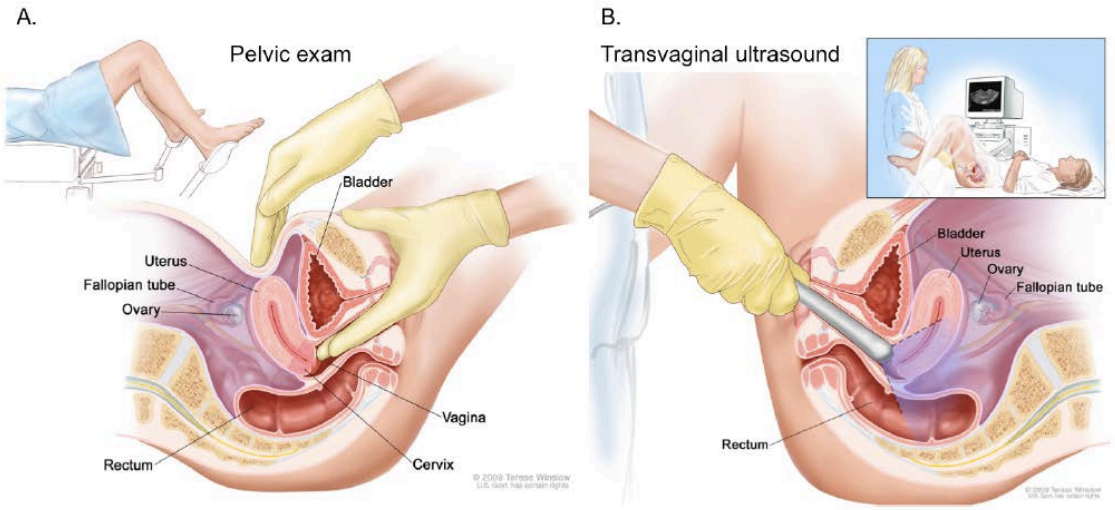


Figure 4. Clinical examination. (A) Pelvic exam. (B) TVS. Image from teresewinslow.com.

Pathological examination

The pathological examination of an endometrial biopsy is the gold standard for EC diagnosis. A small sample of the uterus is taken and observed by a pathologist under the microscope. As the first method of choice, biopsies can be obtained by aspiration,

a minimally invasive, cost-effective, and safe procedure that can be performed in the clinician's office and is well tolerated by patients. These biopsies provide a final diagnosis for 80% of patients with a sensitivity and specificity of 90% and 80%, respectively. When diagnosis cannot be performed, a biopsy guide by hysteroscopy should be performed for a confirmatory diagnosis. Although hysteroscopy has a greater statistical outcome compared to the pipelle biopsy, it is a more invasive technique and requires prior blood testing, anesthesia and a hospital setting, and is associated an increased risk of complications including uterine perforation, infection, and hemorrhage, and consequently, higher healthcare costs. Along with EC diagnosis, the histological type and grade of the tumor will be determined by a specialized pathologist in order to guide the primary treatment [3, 7].

1.4 EC classification

1.4.1 Histological classification

Regarding the histology of the tumor and the characteristics of the cancer cells, we can distinguish between the different EC subtypes and architectural grades. Find this information in Table 1 [10]:

Subtype	Arquitectural grade	Incidence
Endometrioid adenocarcinoma	G1 (glandular normal characteristics with less than a 5% of solid non-squamous regions). G2 (6 to 50% of solid non-squamous areas). G3 (more than 50% of the tumor composed by non-squamous tumoral regions).	80-90%
Papilar serous adenocarcinoma	G3 by definition	1-9%
Cell clear adenocarcinoma	G3 by definition	5-10%
Undiferentiated carcinoma		1-5%
Mixed carcinoma		
Neuroendocrine tumors		

Table 1. Histological classification of the EC. The WHO has described several subtypes of EEC: adenocarcinoma with squamos differentiation, villoglandular carcinoma, secretory carcinoma and also mucinous adenocarcinoma. The other subtypes of EC are considered NEEC [10].

1.4.2 FIGO classification

EC is generally staged according to the International Federation of Gynecology and Obstetrics (FIGO). The FIGO staging is based on information retrieved by the uterine examination after its resection and is a description of the extent to which the cancer has spread and it takes into account the following parameters: tumor size, histology and location, percentage of myometrial invasion, metastasis to proximal organs and pelvic lymph nodes and distal dissemination. This classification, together with the clinical and pathological information, permits to classify patients based on their risk of recurrence [11, 12]. The last modification of FIGO staging was done in 2009 and published in 2010 (Table 2 and Figure 5) [13].

FIGO Stage		Description
Stage I		**Tumor confined to the uterine corpus but not to the uterine serosa.**
	IA	No presence or < 50% of myometrial invasion.
	IB	≥ 50% of myometrial invasion.
Stage II		**Tumoral invasion of the cervical stroma, but not extended beyond the uterus.**
Stage III		Local and/or regional dissemination of the tumor.
	IIIA	Invasion of the serous layer of the uterine corpus and/or adnexae.
	IIIB	Vaginal dissemination.
	IIIC	Metastasis to pelvic and/or para-aortic lymph nodes.
	IIIC1	Positive pelvic nodes.
	IIIC2	Positive para-aortic nodes with or without implication of the pelvis nodes.
Stage IV		**Bladder or intestinal mucosa invasion of the tumor and/or distal metastasis.**
	IVA	Bladder or intestinal mucosa involvement.
	IVB	Distal metastasis including intra-abdominal, extra-abdominal dissemination and/or inguinal lymphatic nodes.

Table 2. FIGO EC staging. Adapted from Pecorelli et al [13].

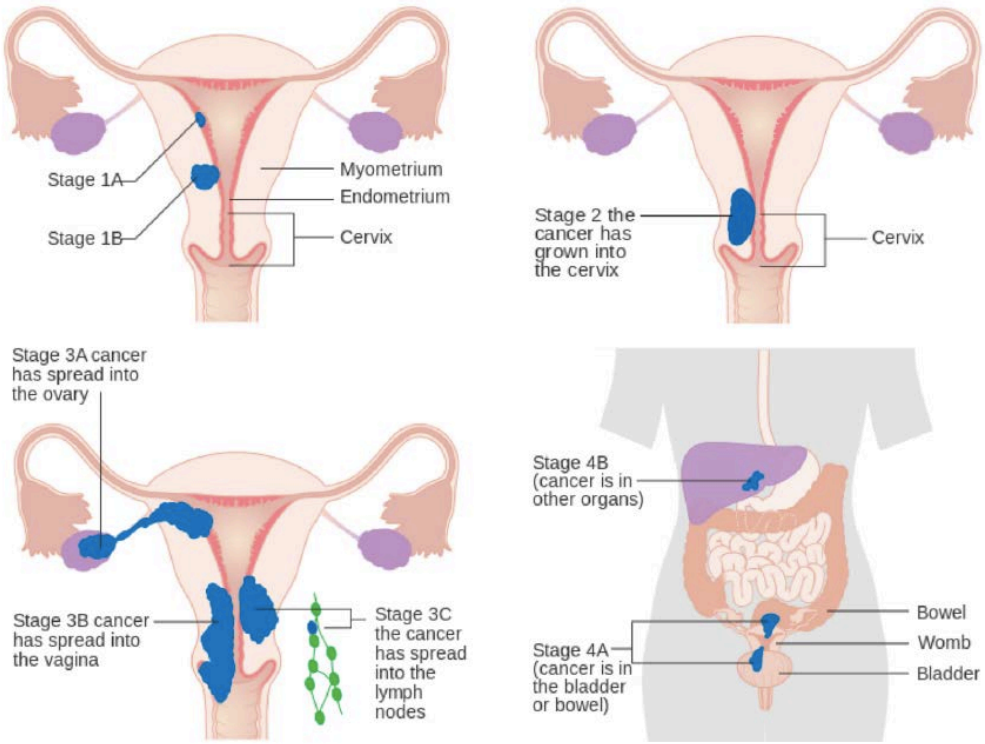


Figure 5. FIGO stages. Image from Cancer research UK (CRUK).

1.5 Treatment

1.5.1 Preoperative risk assessment

According to the recommendations adopted by the ESGO-ESMO-ESTRO consensus conference, an extensive evaluation is mandatory before surgery and it must include family history, list of comorbidities, geriatric assessment, clinical examination, transvaginal or transrectal ultrasound, pathology assessment (type and grade of the tumor) of an endometrial biopsy or curettage sample. The preoperative risk assessment is important to correctly classify patients into those groups of risk for lymphatic dissemination and disease recurrence to define the most appropriate surgical treatment.

On the one hand, endometrial biopsies are obtained by aspiration o guided hysteroscopy and serve to confirm diagnostic sample but also to assess the tumor grade and histological type [14]. Nevertheless, several studies have reported discrepancies between pre- and post-operative biopsies, which could lead to a misclassification and the use of inappropriate therapeutic strategies. Importantly, one factor to take in account in the sample took is intra-tumor genetic heterogeneity that represents a challenge that hampers the correct characterization of tumor samples [15]. The pre-operative staging (defined by the type and grade of the tumor, the percentage of myometrial invasion and the cervical involvement) and the medical condition of the patient will guide the extent of the surgery, which is the primary EC treatment. On the other hand, the magnetic resonance imaging (MRI) is considered the preferred imaging technique for preoperative staging, especially to determine myometrial invasion, lymphatic metastases and cervical involvement although [16].

Emerging molecular imaging techniques (i.e. hybrid PET/MRI) might improve diagnostic accuracy by better soft tissue contrast, multiplanar image acquisition and functional imaging [17].

The definitive staging of the tumor will be determined after surgery and it is known as clinical staging.

1.5.2 Surgery

Surgery is the primary treatment of EC. The surgical intervention typically involves total hysterectomy and removal of both fallopian tubes and ovaries (bilateral salpingo-oophorectomy) and, depending on the tumor characteristics and the stage of the disease, a full pelvic and para-aortic lymphadenectomy will be also required. Surgery also provides valuable information for staging purposes. Conventional oncologic surgery for EC is performed by laparotomy but there is now a movement towards minimally invasive techniques such as laparoscopic approach or robot assisted laparoscopic surgery (RALS) [18]. Minimally invasive surgery is recommended in the surgical management of low-and intermediate-risk EC. The surgical procedures recommended depending on the staging of the tumor are summarized on Table 3 [14].

Preoperative staging		**Recommended surgical procedure**
Stage I	IA; G1–G2	Hysterectomy with bilateral salpingo-oophorectomy
	IA; G3	Hysterectomy with bilateral salpingo-oophorectomy ± bilateral pelvic-para-aortic lymphadenectomy
	IB; G1-G2-G3	Hysterectomy with bilateral salpingo-oophorectomy ± bilateral pelvic-para-aortic lymphadenectomy
Stage II		Radical hysterectomy with bilateral salpingo-oophorectomy and bilateral pelvic-para-aortic lymphadenectomy
Stage III		Maximal surgical cytoreduction with a good performance status
Stage IV	IVA	Anterior and posterior pelvic exenteration
	IVB	Systemic therapeutical approach with palliative surgery
Serous & cell clear		Hysterectomy with bilateral salpingo-oophorectomy, bilateral pelvic-para-aortic lymphadenectomy, omentectomy, appendectomy and peritoneal biopsies

Table 3. Surgical treatment based on tumor staging. G: grade. Adapted from Colombo et al [4].

1.5.3 Adjuvant Treatment

Most of the patients diagnosed with EC fall in the low-risk of recurrence and, consequently, they are solely treated by surgery. However, the disease stage and the recurrence risk of the patient will define the recommended adjuvant treatment [14] (see Table 4).

Preoperative staging		Recommended adjuvant treatment
Stage I	**IA; G1–G2**	Observation
	IA; G3	Observation or vaginal BT (if NPF: pelvic RT and/or adjunctive chemotherapy could be considered)
	IB; G1-G2	Observation or vaginal BT (if NPF: pelvic RT and/or adjunctive chemotherapy could be considered)
	IB; G3	Pelvic RT (if NPF: combination of radiation and chemotherapy could be considered)
Stage II		Pelvic RT and vaginal BT · If grade 1–2 tumor, myometrial invasion <50%, negative LVSI and complete surgical staging: BT alone. · If NPF: chemotherapy + RT
Stage III-IV		Chemotherapy · If positive nodes: sequential radiotherapy · If metastatic disease: chemotherapy – RT for palliative treatment

Table 4. Recommended adjuvant treatment for EC patients. NPF: negative predictive factor; BT: brachytherapy; RT: radiotherapy; LVSI: Lymphovascular space invasion. Adapted from Colombo et al [4].

2. COLORECTAL CANCER

2.1 Epidemiology

Colorectal cancer (CRC) is the second leading cause of cancer death worldwide representing the 9.2% of cancer-related deaths with 881,000 deaths in 2018, and the fourth most commonly diagnosed cancer worldwide with 1,800,00 cases representing the 10.2% of new cancer cases in 2018 (Figure 6) [1].

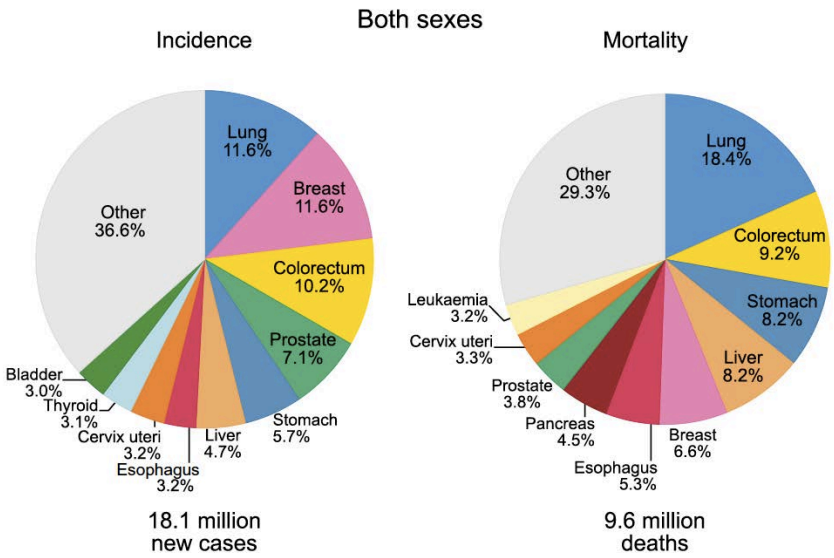


Figure 6. New cases and deaths for cancer. Pie charts represent the distribution of cases and deaths for the 10 most common cancers in 2018 in both sexes. Adapted from Bray et al [1].

Nowadays, 40% of the CRC cases are diagnosed at initials stages when the tumor is localized and the 5-year survival rate is about 90%. Unfortunately, 35% of cases are diagnosed at advanced stages, presenting regional metastasis, and this is associated to a 5-year survival rate of 70%. Moreover, 20% of the patients are diagnosed even at a more advanced stage, i.e. presenting distant metastasis, and for them, the 5-year survival rate decrease drastically to a 14% (Figure 7) [2].

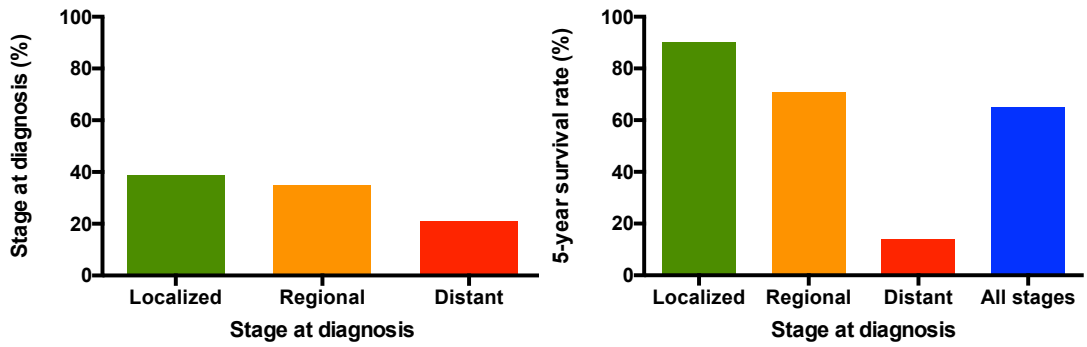


Figure 7. Stage distribution and 5-year relative survival rates of CRC by stage at diagnosis. Adapted from Siegel et al [2].

CRC incidence rate vary widely by world regions; and in fact, this disease can be considered as a marker of socioeconomic development. This is because the rise in incidence is influenced by dietary patterns, obesity and lifestyle factors, whereas the mortality rate declines in more developed countries due to the adoption of cancer screening programs, standardization of preoperative and postoperative care, improvement in surgical techniques and availability of more-effective systemic therapies for early-stage and advanced-stage disease [1].

2.2 Risk and Protective factors

The risk of developing CRC depends on factors which can be classified into lifestyle or behavioral factors (such as smoking, high red meat consumption, obesity, physical inactivity) and genetically determinant factors. Age is considered the major unchangeable risk factor for sporadic CRC: nearly 70% or patients with CRC are over 65 years, and this disease is rare before 40 years even if data from western registries showed an increased incidence in the 40-44 years group and a decrease in the oldest groups [19]. CRC occurs sporadically in most of the cases, but also, it can be inherited. This is estimated to occur in 5%–10% of cases. Diet is definitely the most important exogenous factor identified in the etiology of CRC up to now, there are convincing evidences that increased consumption of red and processed meat,

alcoholic drinks, body and abdominal fatness, all increase the risk for CRC, especially in taller people [20]. Regarding the non-dietary factors, the most important are: smoking tobacco, which has consistently been associated with large colorectal adenomas (generally accepted as precursors for cancer) and has been potentially attributable cause of one in five CRC in United States of America (USA); inflammatory bowel diseases (Crohn's disease and ulcerative colitis); patients who have had previous malignant disease are also at great risk of developing a second colorectal tumor; and the metabolic syndrome (high blood pressure, increased waist circumference, hypertriglyceridaemia, low levels of high-density lipoprotein cholesterol or diabetes/ hyperglycaemia). As genetic vulnerability to colon cancer has been attributed to either polyposis or non-polyposis syndromes.

Some preventive factors to avoid the risk of developing CRC have also been identified. Based on significant evidence, postmenopausal estrogen plus progesterone hormone use decreased the incidence of CRC but a non-comparable benefit was demonstrated for estrogen alone. Moreover, there is evidence from three randomized trials that aspirin significantly reduced the recurrence of sporadic adenomatous polyps whereas there was evidence from short-term trials to support regression, but not elimination or prevention, of colorectal polyps in familial adenomatous polyposis. Recently, the World Cancer Research Fund International (WCRF) and the American Institute for Cancer Research (AICR) in their extensive report on diet, physical activity and prevention of cancer have concluded that CRC is mostly preventable by appropriate diet and associated factors [21]. There is convincing evidence that increasing dietary fiber and reducing red and processed meat consumption and alcoholic drinks as well as regular physical exercise reduced the risk of CRC [20].

2.3 Diagnosis

CRC may be diagnosed when a patient presents with symptoms or as the result of screening programs.

2.3.1 Signs and symptoms

Symptoms are associated with relatively large tumors and/or advanced disease stages, and are generally not specific for colon cancer. Change in bowel habits, general or localized abdominal pain, weight loss without other specific causes, weakness, iron deficiency and anemia are the most common symptoms, and depends on the location and stage of the primary tumor; they are associated with worse prognosis and their number (but not their duration) is inversely related to survival [22]. In symptomatic patients, colonoscopy is the preferred method of investigation, but other endoscopic methods are also available or being developed. A systematic review and meta-analysis of the published literature were carried out to assess the diagnostic accuracy (sensitivity, specificity, and positive and negative ratios) of alarm features in predicting large bowel cancer, resulting in a pooled prevalence of CRC of 6% (95% CI: 5% to 8%) in >19,000 cases, and only dark red rectal bleeding and abdominal mass had a specificity of >95%, suggesting that the presence of either characteristic strongly indicates a diagnosis of CRC [23]. Although the predictive value of symptoms for the presence of CRC is limited, they do warrant further clinical evaluation.

2.3.2 Screening

Because early cancer produces no specific symptoms, aggressive efforts at detection through screening programs are essential. The aim of screening programs is to detect a pre-cancer condition in a healthy population, as well as very early-stage malignancies which can be treated with a clearly curative intention [24]. In CRC, screening programs aimed to detect adenomatous polyp, as this is the most common premalignant lesion in CRC, as well as early CRC. For population screening a range

of methods (Table 5) can be used for primary assessment followed by colonoscopy in case of positive test [21].

Test	Advantages	Disadvantages
gFOBT	Cheap Low screenee burden Reasonable uptake	Limited sensitivity for advanced neoplasia Need for short screening intervals No effect on the incidence of CRC Qualitative, not automated Multiple sampling Moderate positive predictive value
FIT	Cheap Low screenee burden Quantitative, automated Single sample Sensitive for CRC Highest uptake Effect on incidence and mortality	Limited sensitivity for advanced adenoma Moderate positive predictive value Repeated screening needed (interval can be longer than for gFOBT) Temperature-dependent performance
Sigmoidoscopy	Sensitive for distal advanced neoplasia Long screening interval Effect on incidence and mortality	Low uptake Expensive Moderately sensitive for proximal advanced neoplasia
Colonoscopy	Sensitive and specific Long screening interval Effect on incidence and mortality	Low uptake Expensive Burdensome Associated with complications
CT colonography	Sensitive and specific Long screening interval Likely effect on incidence and mortality	Low uptake Expensive Need for repeated lavage in case of advanced neoplasia Radiation exposure Burdensome
Multi-target faecal DNA test	Sensitive and specific	Untake unknown Expensive Lack of prospective date

Table 5. Key performance indicators for organized screening with different modalities. FIT: faecal immunochemical test; gFOBT: guaiac faecal occult blood test. Adapted from Kuipers et al [25].

FOBT is the most extensively scrutinized method and has been shown to reduce mortality by up to 25% CRC among those attending at least one round of screening. The Advisory Committee on Cancer Prevention suggested that if screening programs for CRC are implemented, they should use the FOBT, whereas colonoscopy should be used for the follow-up of test-positive cases, and that the screening should be offered to men and women aged 50 years until 74 years with an intervals of 1–2 years [21].

The following individuals are considered at high risk of colon cancer and must be actively screened and, in case of inherited syndromes, also referred for genetic counselling:

- Personal history of adenoma, colon cancer, inflammatory bowel disease
- Significant family history of CRC or polyps
- Inherited syndrome (5-10% of all colon cancers) such as familial adenomatous polyposis coli and its variants (1%), Lynch-associated syndromes, Turcot-, Peutz-Jeghers- and MUTYH-associated polyposis syndromes [19].

Taking into account the impact of CRC on the EU population, with the associated use of expensive adjuvant and palliative therapies, organizing CRC mass screening is a priority which will contribute to reduce incidence, improve prognosis and decrease treatment related morbidity because of stage migration. A population-based cancer registry is a necessity to monitor investments and quality measure, either by implementing primary prevention, population based screening or by improved diagnosis and clinical care for CRC patients [20].

2.3.3 Diagnostic procedure

Clinical examination

A complete colonoscopy up to the cecum, coupled with biopsy for histopathological examination, is considered the gold standard to diagnose colorectal lesions, in view of its high diagnostic performance. Endoscopy is the main procedure for diagnosis and can be carried out by either sigmoidoscopy (as >35% of tumors are located in the rectosigmoid) or (preferably) a total colonoscopy. However, a substantial proportion of patients will have an incomplete colonoscopy due to poor bowel preparation, poor patient tolerance, obstruction or other technical difficulties. In these cases, additional computed tomography colonography (CTC) can contribute to the CRC diagnosis as a potential alternative to the endoscopy. CTC does not offer the opportunity of taking biopsies or immediate polypectomy and the patient needs to return for a colonoscopy, in case of detected lesions [26].

- Colonoscopy

  Colonoscopy is the gold standard for the diagnosis of CRC with a high diagnostic accuracy than can assess the location of the tumor. The technique can enable simultaneous biopsy sampling and, hence, histological confirmation of the diagnosis and material for molecular profiling. Colonoscopy is also the only screening technique that provides both a diagnostic and therapeutic effect because removal of adenomas using endoscopic polypectomy can reduce cancer incidence and mortality. Over the past 20 years, the image quality of colonoscopy has markedly improved, from original fiber-optic to videochip endoscopes which have improved over the years leading to higher resolution and wider angle of view. The invasive nature of colonoscopy poses a burden to screenees and patients, which might affect participation in screening programs. In recent years, several alternative diagnostic methods have been introduced, such as capsule endoscopy and biomarker tests [24, 27].

- Capsule endoscopy

  Capsule endoscopy uses a wireless capsule device that is swallowed by the screenee and enables examination of almost the entire gastrointestinal tract without the use of conventional endoscopy. Capsule endoscopy is useful in diagnosing adenomas and CRC. The European Society for Gastrointestinal Endoscopy (ESGE) guideline for colon capsule endoscopy recommends capsule endoscopy as a feasible and safe tool for the visualization of the colonic mucosa in patients who have undergone no or incomplete colonoscopies. The indications for capsule endoscopy are at this moment limited to patients who refuse conventional colonoscopy and to those in whom a complete colonoscopy is not possible for anatomical reasons. The presence of a stenosis is a contraindication for capsule endoscopy as it could lead to capsule retention [28, 29].

- Computed tomography colonography

  CTC uses low-dose CT scanning to obtain an interior view of the colon implemented for colonoscopy screening. CTC requires full bowel preparation (that is, clearance of the bowel), air inflation and a change in position of the patients during the examination. The discomfort to the screenee undergoing CTC is similar to colonoscopy in experienced hands, particularly because of the need for substantial bowel insufflation, but it has the advantage of obviating the use of sedation and can be used as part of the staging procedure in a confirmed case of CRC. However, CTC has low sensitivity for small and flat lesions. The costs of CTC and the need for further investigation in a subset of screenees limit the usefulness of this method for population screening in most countries [30, 31, 32].

- Biomarkers of CRC

  Molecular detection of CRC offers a non-invasive test that is appealing to patients and clinicians as samples of multiple patients can be analyzed in a batch. The ideal molecular marker should be highly discriminating between cancer and advanced adenomas from other lesions, be continuously released into the bowel lumen or circulation, and disappear or reduce after the lesion is removed or treated. Indeed, assays using proteins, RNA and DNA in the blood, stool and urine have been developed but with varying degrees of success (Table 6) [25].

Gene of Biomarker	Frequency (%)	Predictive?	Prognostic?	Diagnostic?
APC	40-70	No	No	Familial adenomatous polyposis
ARID1A	15	No	No	Not appliable
CTNNB1	1	No	No	No
DCC	9 (mutation); 70 (LOH)	No	Possible	No
FAM123B	10	No	No	No
FBXW7	20	No	No	No
PTEN	10 (mutation); 30 (loss of expression	Possible	No	Cowden syndrome
RET	7 (mutation); 60 (methylations)	No	No	No
SMAD4	25	Possible	Possible	Juvenile polypsis
TGFBR2	20	No	No	No
TP53	50	Possible	Possible	Li-Fraumeni syndrome
BRAF	ag-28	Probable	Probable	Lynch syndrome
ERBB2	35	No	No	No
GNAS	20	No	No	No
IGF2	7 (mutation); 10 (mhylations)	No	No	No
KRAS	40	Yes	Possible	Not appliable
MYC	2 (mutation); 10 (CNV gain)	No	No	No
NRAS	2	Yes	No	No
PIK3CA	20	Probable	Possible	No
RSPO2 and RSPO3	10	No	No	No

SOX9	9 (mutation); 5 (CNV gain)	No	No	No
TCF7L2	10	No	No	No
Chromosome instability	70	Probable	Probable	No
CpG island methylator phenotype	15	Probable	Probable	No
Microsatellite instability	15	Probable	Yes	Lynch syndrome
Mismatch-repair genes	1-15	Possible	Probable	Lynch syndrome
SEPT9	90	No	No	Serum-based assay for cancer detection
VIM, NDRG4 and BMP3	75	No	No	Stool-based test for early detection
18qLOH	50	Probable	Probable	No

Table 6. Common genetic and epigenetic alterations in CRC. Adapted from Adapted from Kuipers et al [25].

2.4 CRC classification

2.4.1 Histological classification

Regarding the histology of the tumor and the characteristics of the cancer cells, we can distinguish between the different CRC subtypes (see Table 7).

Subtype	Incidence
Adenocarcinoma	95%
Others	
Carcinoid tumors	
Gastrointestinal stromal tumors	5%
Lymphomas	
Sarcomas	

Table 7. Histological classification of CRC.

2.4.2 Staging classification

Staging is crucial to ensure a correct treatment strategy. The standard assessment should include the morphological description of the specimen, surgical procedure carried out, definition of tumor site and size, presence or absence of macroscopic tumor perforation, histological type and grade, extension of tumor into the bowel wall and adjacent organs (T stage), distance of cancer from resected margins (proximal, distal and radial), presence or absence of tumor deposits, lymphovascular and/or perineural invasion, presence of tumor budding, site and number of removed regional lymph nodes and their possible infiltration by cancer cells (N stage), and finally the possible involvement of other organs (e.g. liver) if submitted for removal or biopsy (M stage) [33].

The pathological stage must be reported according to the American Joint Cancer Committee (AJCC)/ Union for International Cancer Control (UICC) TNM classification, 7th edition (Table 8) and these stages are combined into an overall stage definition (Table 9), which provides the basis for therapeutic decisions. Although classification according to TNM and UICC stage provides valuable prognostic information and guides therapy decisions, the response and outcome of individual patients' therapy is not predicted [34].

	Description
T stage	
Tx	No information about local tumor infiltration available
Tis	Tumor restricted to mucosa, no infiltration of lamina muscularis mucosae
T1	Infiltration through lamina muscularis mucosae into submucosa, no infiltration of lamina muscularis propia
T2	Infiltration into, but not beyond, lamina muscularis propia
T3	Infiltration into subserosa or non-peritonealised pericolic or perirectal tissue, or both; no infiltration of serosa or neighbouring organs
T4a	Infiltration of the serosa
T4b	Infiltration of neighboring tissues or organs
N stage	
Nx	No information about lymph node involvement available
N0	No lymph node involvement
N1a	Cancer cells detectable in 1 regional lymph node
N1b	Cancer cells detectable in 2-3 regional lymph nodes
N1c	Tumor satellites in subserosa or pericolicor perirectal fat tissue, regional lymph nodes not involved
N2a	Cancer cells detectable in 4-6 regional lymph nodes
N2b	Cancer cells detectable in 7 or greater regional lymph nodes
M stage	
Mx	No information about distant metastases available
M0	No distant metastases detectable
M1a	Metastasis to 1 distant organ or distant lymph nodes
M1b	Metastasis to more than 1 distant organ or se of distant lymph nodes or peritoneal metastasis

Table 8. Classification of CRC according to local invasion depth (T stage), lymph node involvement (N stage), and presence of distant metastases (M stage). Adapted from Sobin et al [34].

Stage	T	N	M
Stage 0	Tis	N0	M0
Stage I	T1/T2	N0	M0
Stage II	T3/T4	N0	M0
IIA	T3	N0	M0
IIB	T4a	N0	M0
IIC	T4b	N0	M0
Stage III	Any	N+	M0
IIIA	T1-T2	N1	M0
	T1	N2a	M0
IIIB	T3-T4a	N1	M0
	T2-T3	N2a	M0
	T1-T2	N2b	M0
IIIC	T4a	N2a	M0
	T3-T4a	N2b	M0
	T4b	N1-N2	M0
Stage IV	Any	Any	M+
IVA	Any	Any	M1a
IVB	Any	Any	M1b

Table 9. Stage classification of CRC. Adapted from Sobin et al [34].

2.5 Treatment

CRC includes colon and rectal cancers, which are two distinct cancers requiring different approaches, also depending on their stage. A general improvement of CRC treatment is emerging but the outcome after CRC treatment show huge differences depending on the countries [25]. Patients with CRC should be assessed by a multidisciplinary team to decide upon the best treatment strategy[35].

2.5.1 Surgery

Surgery is the mainstay curative treatment for patients with non-metastasized CRC. However, outcome is strongly related to the quality of surgery, the quality of preoperative staging and treatment selection. The dissection should ideally follow the embryological anatomical planes to ensure that the tumor and its principal zone of lymphatic spread are removed. Special attention should be given to the

circumferential surgical resection margins. In more-advanced cases of rectal cancer, neoadjuvant treatment can reduce tumor load and even tumor stage, and might be necessary to optimize the chances for a successful resection. Thus, a multidisciplinary approach before beginning treatment, based on adequate staging information, is mandatory [25].

Rectal cancer

The standard surgical procedure for the treatment of rectal cancer is total mesorectal excision. Complete removal of the mesorectum is important because it contains most of the involved lymph nodes and tumor deposits. Several studies have shown the importance of achievement of clear lateral margins (circumferential margin) generally defined as a distance of greater than 1 mm between the tumor border and the resection margin. Patients with involved circumferential margin have increased risk of local recurrence and development of distant metastases. The plane of the mesorectal fascia is used for resection, but resection has to be extended laterally if the tumor spreads beyond the fascia [36].

Colon cancer

In colon cancer surgery, the tumor and the corresponding lymph vessels are removed. The extent of surgery is predetermined by the tumor localization and the supplying blood vessels. Some experts have proposed complete mesocolic excision for colon cancer surgery, with separation of the mesocolic plane from the parietal plane and central ligation of the supplying arteries and draining veins. Complete mesocolic excision results in resection of increased mesocolon and lymph nodes [37]. Further data for the risks and benefits of complete mesocolic excision are needed. Open surgery used to be the only option available; however, laparoscopic resection has become an alternative. Several studies have shown that laparoscopic resection of colorectal cancer achieves the same long-term results as open surgery, and is associated with a reduced number of patients requiring blood transfusions, faster

return of bowel function and a shorter duration of hospital stay. However, operating times are longer and operative costs are higher in laparoscopic surgery [38]. In the emergency setting, when presenting symptoms of obstructions, perforation and bleeding, segmental colectomy for resection of the tumor, with or without fecal diversion is indicated. In presence of unresecable metastatic lesions or as initially palliation of obstructing CRC, the use of self-expandable metal stents (SEMS) is gaining wide acceptance, also to allow a quick start of neoadjuvant chemotherapy or chemoradiation. The colonic stent insertion effectively decompresses the obstructed colon and surgery can be performed electively at a larger stage avoiding a derivate ostomy, whenever possible [39].

2.5.2 Neoadjuvant therapy

Rectal cancer

With the introduction of total mesorectal excision, the rate of local recurrences after surgery of rectal cancer has fallen substantially and nowadays the question remain in which patients and how must receive neoadjuvant therapy.

- Stage I patients should not be given any treatment in addition to surgery because the local recurrence rate is low and the benefit from neoadjuvant treatment very small.
- Stage II patients presents an unclear benefit of neoadjuvant treatment.
- Stage III patients benefit from additional treatment.
- Advanced T3 infiltrating the mesorectal fascia and T4 patients are generally accepted for neoadjuvant treatment.

Colon cancer

Data for the role of neoadjuvant treatment in locally advanced colon cancer are scarce. Some studies have showed that preoperative chemotherapy is feasible, with acceptable toxicity and perioperative morbidity, and statistically significantly

increased the rate of R0 resections. However, further data from randomized trials are needed to extract definitive conclusions [35].

2.5.3 Adjuvant therapy

- Stage II colon cancer is associated with statistically significantly better disease-free survival and overall survival than stage III colon cancer. Accordingly, the survival benefit from adjuvant chemotherapy seems to be reduced, and thus is generally recommended only for patients at high risk of relapse.

- Stage III colon cancer patients have a risk of recurrence ranging between 15% and 50% and that is the reason why adjuvant chemotherapy is recommended after curative resection for all of those patients.

Regimens containing fluorouracil reduce recurrence rate and increase overall survival and capecitabine, an oral prodrug of fluorouracil, can be used with comparable efficacy. Several large prospective trials have investigated the addition of oxaliplatin to fluorouracil and capecitabine that increased the absolute 5-year disease-free survival and the overall survival in patients with stage III colon cancer. However, some studies suggest that this benefit might be limited to patients younger than 65 years or younger than 70 years. In large randomized trials, the addition of bevacizumab or cetuximab to an oxaliplatin containing regimen did not show any benefit on disease-free survival. Additionally, the use of irinotecan combined with fluorouracil did not show any benefit and was associated with increased toxic effects [35].

3. LUNG CANCER

3.1 Epidemiology

Lung cancer (LC) remains the leading cause of cancer incidence and mortality worldwide. With 2.1 million new LC cases (11.6% of the total cases) and 1.8 million deaths (18.4% of the total cancer deaths) predicted in 2018 (see Figure 6), the disease has become an epidemic as incidence rates and LC deaths have risen dramatically over the last century representing close to 1 in 5 cancer deaths.

In contrast to the steady increase in survival observed for most cancer types, advances have been slow for LC. Unfortunately, the mortality rate closely parallels the incidence rate for LC because of persistently low patient survival. Among the reasons of its high mortality is the fact that 57% of the patients of the cases are diagnosed at a late stage in which the 5-year survival is 5%. Advances in treatment, including surgical, medical, and radiotherapeutic interventions, have provided little improvement in the long-term survival rate of patients diagnosed with primary malignancies. Despite that, there still exist a 16% of patients that are diagnosed at initial stages when the tumor is still confined, and the 5-year survival rate associated is quite high (56%)[2] (Figure 9). Diagnosis at early stages of the disease is limited by the fact that LC symptoms occur late in the disease, and there is not a screening program. Improvement in early diagnosis could therefore have tremendous impact in decreasing mortality rates with timely therapeutic interventions and disease management.

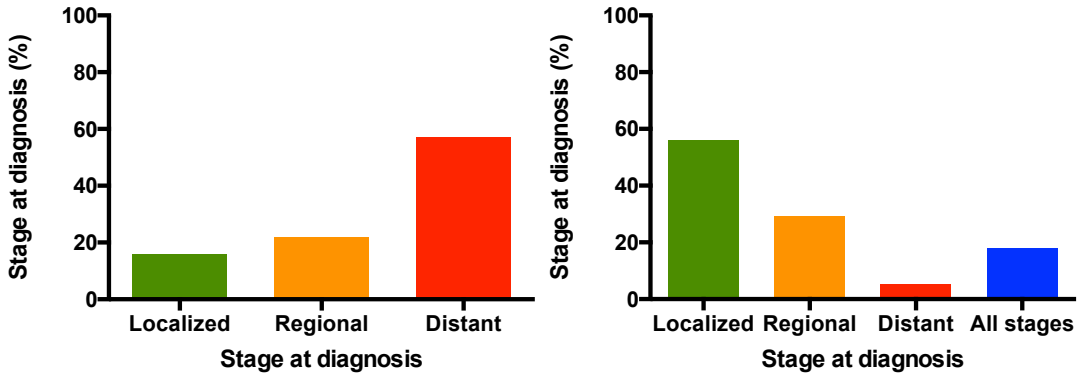


Figure 8. Stage distribution and 5-year relative survival rates of LC by stage at diagnosis. Adapted from Siegel et al [2].

3.2 Risk and Protective factors

Genetic, behavioral and environmental factors are involved in the pathogenesis of LC (Figure 10). Tobacco cigarette smoking is the predominant risk factor of LC and its use causes from 80% to 90% of all LCs. There is a direct dose-response relationship between the number of cigarettes smoked and the risk of LC. Although the rate of smoking has declined in both sexes, millions of individuals continue to smoke and thus are at a higher risk for this malignancy and that is why smoking prevention will undoubtedly remain the primary and most important intervention to further decrease LC mortality [40, 41, 42]. Moreover, a growing number of incident LC are occurring in never-smokers. This group comprises 15 to 25% of the LC population and are linked with environmental factors, including second-hand tobacco smoke, outdoor and indoor air polution, and radon exposure [43, 44]. Radon, a naturally occurring radioactive gas, is recognized as the second leading cause of LC and the exposure to this gas progeny may account for 10% of all LC deaths [45]. Increasingly, LC is more likely to occur in poorer and less-educated populations, primarily reflecting the increasing gradient of smoking with socioeconomic indicators that include income, education, and occupation. Lifestyle factors other than cigarette smoking, such as diet and exercise, have been extensively investigated for a potential role in influencing LC risk. LC is also causally associated with many

workplace exposures and, in addition, intrinsic host factors can affect susceptibility to developing LC [41].

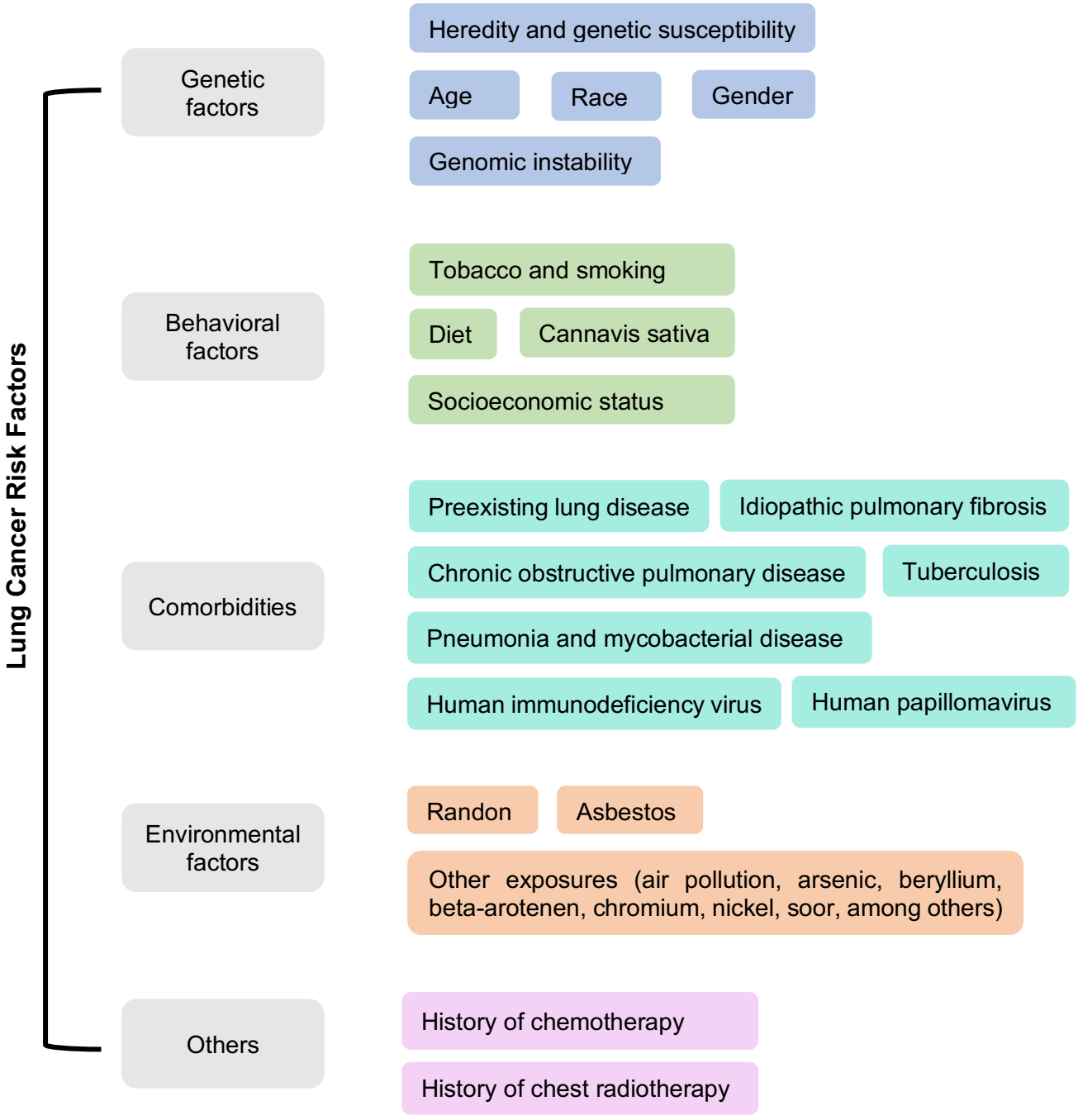


Figure 9. Risk factors of LC.

As protective factors, there are several ways through which the incidence of LC might be reduced:

- Tobacco cessation and Tobacco-control legislation

  Never smoking is the best way to prevent LC, and smoking cessation is helpful. Legislation such as smoking bans in public buildings, prohibiting marketing of tobacco products to minors, and taxation of tobacco products likely play a role in decreasing tobacco use [46].

- Vaccination

  If future research reveals a stronger and contributory relationship between Human Papillomavirus (HPV) and LC, the vaccination program may be very helpful, and similar strategies could be used as in other cancer also associated to this virus.

- Radon control

- Diet

  Proper diet constitutes an important contribution to the prevention of LC and offers a widely available way, through education, to reduce the risk of cancer in the general population [42].

3.3 Diagnosis

3.3.1 Signs and symptoms

Clinical manifestations of LC are diverse and patients are mostly asymptomatic at early stages. Further, symptoms are subtle and non-specific, resembling more common benign etiologies. Accordingly, LC is more frequently diagnosed at advanced stages when patient prognosis is poor. Symptoms can be caused by the

primary tumor (e.g., cough, hemoptysis); intrathoracic spread (e.g., horner syndrome, superior vena cava obstruction); and distant metastases (e.g., bone pain). Symptoms can also be caused by paraneoplastic syndromes, such as the syndrome of inappropriate antidiuretic hormone. These symptoms are a result of ectopic production of hormones from the tumor or the body's reaction to the tumor (e.g., digital clubbing). Most data about symptoms at presentation of LC are from referral centers, making extrapolation to the primary care setting difficult. Two individual symptoms that significantly increase the likelihood of LC are digital clubbing and hemoptysis. Other independent predictors of LC include loss of appetite, weight loss, fatigue, dyspnea, chest or rib pain, and an increasing number of visits to evaluate persistent cough [47, 48, 49].

3.3.2 Screening

LC symptoms occur late in the disease, so the majority of patients with LC present with advanced disease. Unfortunately for those patients, the disease will not be curable with currently available therapies. Therefore, early detection might be a valuable approach to detect the disease at an earlier, asymptomatic and potentially curable stage. Although it would be ideal if all individuals could be screened, it is neither realistic nor advantageous to do so. Over the last years, many trials have failed to show benefit if periodical chest radiograph (CXR) and/or sputum cytology were used; screening by these techniques is therefore not recommended [50].

The National Lung Cancer Screening Trial (NLST) is the only trial that has shown a decrease in mortality resulting from LC screening [51]. Researchers demonstrated a 20% reduction in LC mortality by annual low-dose computed tomography (LDCT) compared with CXR with a decrease in overall mortality by 6.7% [52]. Although the NLST did show an important reduction in mortality rates in LC-related deaths, several concerns should be addressed before screening becomes standard care. The risks of lung screening include false-negatives and false-positives, radiation exposure,

overdiagnosis of incidental findings, futile detection of aggressive disease, anxiety, unnecessary testing, complications from diagnostic workup, and financial costs [53]. Nevertheless, for part of the Western world this positive trial has resulted in guidelines for screening within high-risk groups. US preventive Services Task Force recommends LC screening with LDCT in adults of age 55 to 80 years who have a 30 pack-year smoking history and are currently smoking or have done it within the past 15 years [48]. As the tools for LC screening techniques continue to improve, more smokers and ex-smokers are diagnosed with small lung tumors. Considering the magnitude of this global public-health problem, the dialogue on facilitating progress on this complex but vital research agenda must be international, and it could build on the useful process that has begun [54].

3.3.3 Diagnostic procedure

Clinical examination

The initial evaluation of a patient with suspected LC includes:

- History and physical examination

  It is performed in order to know the probabilities of comorbidities, the functional status of the patient, his/her preferences, the probability of cancer, and the evidence of metastatic disease [49].

- Physiologic and laboratory assessment

  This exam might include a complete blood count, measurement of alkaline phosphatase, hepatic transaminase, and calcium levels and chemistries, among others [47].

- Imaging including CXR and sputum cytology

  These techniques are invasive and are usually performed after development of symptoms which is most often encountered at late stages. In 1990, spiral computed tomography (CT) was introduced as a promising technique for early

LC detection. It is more sensitive than CXR and allows imaging of tumors that are less than several centimeters in diameter. However, despite its success and sensitivity, it suffers from serious limitations because represents a high rate of false-positive cases detection [55].

Pathology examination

The current standard of care for diagnosing and treatment-decision making is based on the identification of malignant cells on a tissue-biopsy sample. The collection of the sample could be challenging in small or peripherally located LC. A variety of diagnostic methods are available that yield cytology samples or small biopsies and the choice of procedure depends on the type, location, and size of the tumor; comorbidities; and accessibility of metastases (Table 10). In general, the least invasive method possible should be used and if the procedure fails to obtain tissue, a more invasive method is then performed. Conventional bronchoscopy works best for central lesions, whereas CT-guided transthoracic needle aspiration is typically the first-line method for peripheral lesions. Endobronchial ultrasound and electromagnetic navigation are some of the newer procedures that may increase the diagnostic yield of bronchoscopy for select patients with mediastinal or peripheral lesions [47].

Method	Comments
Biopsy or fine-needle aspiration of accessible metastasis or lymph node	Used in the presence of palpable lymph nodes
Conventional bronchoscopy brushings and washings	High sensitivity for central lesions, much lower sensitivity for peripheral lesions
Computed tomography-guided transthoracic needle aspiration	Good for peripheral lesions seen on computed tomography, associated with pneumothorax, lower sensitivity for smaller lesions
Transbronchial needle aspiration	Indicated for central lesions
Electromagnetic navigation bronchoscopy	Improved diagnostic yield for bronchoscopy of peripheral lesions, requires advanced training beyond skill of most bronchoscopists
Endobronchial ultrasound-guided transbronchial needle aspiration	Best for paratracheal, subcarinal, and perihilar nodes, lower sensitivity for peripheral lesions, requires advanced training beyond skill of most bronchoscopists
Pleural biopsy	Used with pleural effusion and if pleural fluid cytology findings are negative
Sputum cytology	Indicated for central lesions, noninvasive, follow-up testing required if findings are negative
Thoracentesis (pleural fluid cytology)	Easily accessible if present, ultrasound guidance improves yield and decreases risk of pneumothorax, second sample increases diagnostic yield
Video-assisted thoracic surgery	Used for a small single high-risk nodule

Table 10. Methods for tissue diagnosis of LC. Adapted from Alavanja et al [47].

The most common diagnostic test for LC is fiberoptic bronchoscopy, often extended with evaluation of regional lymph nodes by endobronchial ultrasound (EBUS) and/or endoscopic ultrasound (EUS) [50] .

3.4 Lung cancer classification

3.4.1 Histological classification

Regarding the histology of the tumor and the characteristics of the cancer cells, we can distinguish between the following LC types (Table 11) [56]:

Subtype	Incidence
Small cell carcinoma (SCLC)	
Pure small cell lung carcinoma Combined small cell/ non-small cell lung carcinoma	15%
Non-small cell carcinoma (NSCLC)	
Carcinoid tumor Carcinomas with pleomorphic, Sarcomatoid, or scomatous elements Large cell carcinoma Squamous cell carcinoma Adenocarcinoma	75-80%

Table 11. Histological classification of the LC.

3.4.2 Staging classification

Clinical staging is based on all information obtained before treatment, including findings from CT and position emission tomography and invasive staging such as mediastinoscopy. Pathologic staging is performed after surgical resection and may upgrade or downgrade the clinical staging. LC is staged according to the 7th editions TNM staging system [34] (see Table 12 and 13) as the Figure 11 shows [57, 58].

	Description
T stage	
Tx	Primary tumor cannot be assessed, or tumor proven by the presence of malignant cells in sputum or bronchial washings but not visualized by imaging or bronchoscopy
T0	No evidence of primary tumor
Tis	Carcinoma in situ
T1	Tumor 3 cm or less in greatest dimension, surrounded by lung or visceral pleura, without bronchoscopic evidence of invasion more proximal than the lobar bronchus
T1a	Tumor 2 cm or less in greatest dimension
T1b	Tumor more than 2 cm but not more than 3 cm in greatest dimension
T2	Tumor more than 3 cm but not more than 7 cm
T2a	Tumor more than 3 cm but not more than 5 cm in greatest dimension
T2b	Tumor more than 5 cm but no more than 7 cm in greatest dimension
T3	Tumor more than 7 cm or one that directly invades any of the following: chest wall (including superior sulcus tumors), diaphragm, phrenic nerve, mediastinal pleura, parietal pericardium; or tumor in the main bronchus less than 2 cm distal to the carina1 but without involvement of the carina; or associated atelectasisor obstructive pneumonitis of the entire lung or separate tumor nodule(s) in the same lobe as the primary
T4	Tumor of any size that invades any of the following: mediastinum, heart, great vessels, trachea, recurrent laryngeal nerve, esophagus, vertebral body, carina; separate tumor nodule(s) in a different ipsilateral lobe to that of the primary
N stage	
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in ipsilateral peribronchial and/or ipsilateral hilar lymph nodes and intrapulmonary nodes, including involvement by direct extension
N2	Metastasis in ipsilateral mediastinal and/or subcarinal lymph node(s)
N3	Metastasis in contralateral mediastinal, contralateral hilar, ipsilateral or contralateral scalene, or supraclavicular lymph node(s)
M stage	
M0	No distant metastasis
M1	Distant metastasis
M1a	Separate tumor nodule(s) in a contralateral lobe; tumor with pleural nodules or malignant pleural or pericardial effusion
M1b	Distant metastasis

Table 12. Classification of LC according to local invasion depth (T stage), lymph node involvement (N stage), and presence of distant metastases (M stage). Adapted from Sobin et al [34].

Stage	T	N	M
Stage 0	Tis	N0	M0
Stage IA	T1a, b	N0	M0
Stage IB	T2a	N0	M0
Stage IIA	T2b	N0	M0
	T1a, b	N1	M0
	T2a	N1	M0
Stage IIB	T2b	N1	M0
	T3	N0	M0
Stage IIIA	T1a, b, T2a, b	N2	M0
	T3	N1, N2	M0
	T4	N0, N1	M0
Stage IIIB	T4	N2	M0
	Any T	N3	M0
Stage IV	Any T	Any N	M1

Table 13. Stage classification of LC. Adapted from Sobin et al [34].

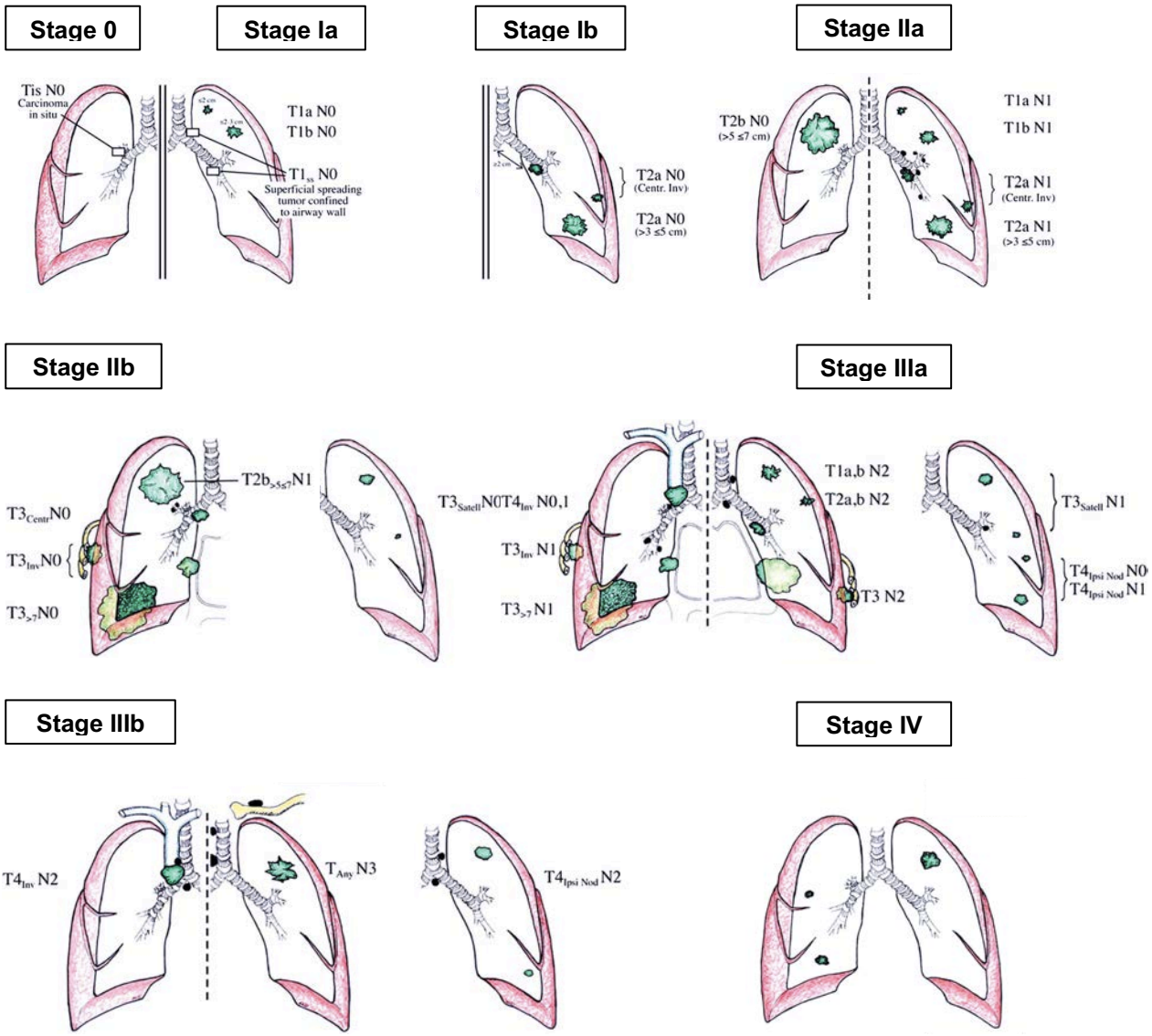
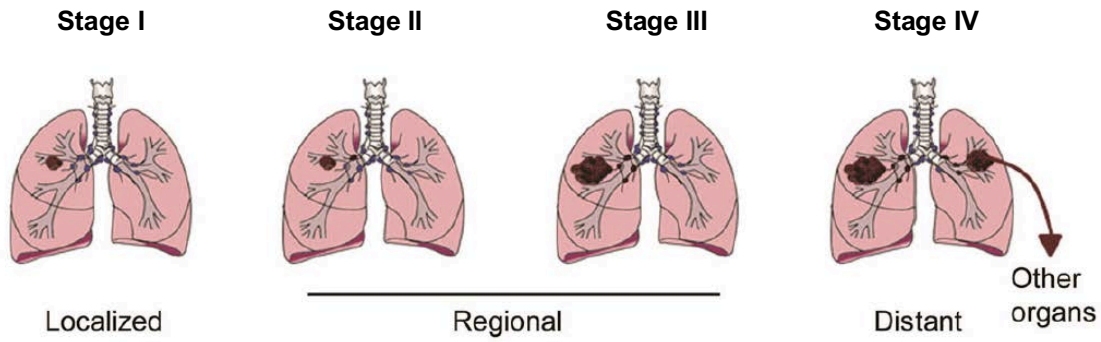


Figure 10. Graphic illustration of LC stages. Adapted from Detterbeck et al [58].

3.5 Treatment

Standard treatments for lung cancer include surgery, platinum based chemotherapy, radiotherapy, combined chemo radiotherapy, and targeted therapy, either alone or in combination (Figure 12). Surgery to remove the tumor and the nearby lymph nodes is the most consistent and successful treatment at early stage. Tumors can be removed by: anatomic segmentectomy, lobectomy, or pneumonectomy. When surgery is no longer an option, RT and/or chemotherapy may be suggested. Although external beam RT is normally used to treat all types of lung cancer, poor prognosis

is still a major problem in NSCLC. For patients with advanced and metastatic NSCLC, chemotherapy is the main therapeutic strategy [55].



Treatment approaches	Surgery	Preoperative chemotherapy and Surgery	Localized treatment and Adjuvant chemotherapy	Systematic radiation therapy Chemotherapy for palliation of symptoms

Figure 11. Treatment of LC at different stages. Adapted from Mehta et al [55].

4. BIOMARKERS

4.1 Biomarker definition

In 1998, the working Group of the National Institute of Health defined a biomarker as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. An optimal biomarker should be easily obtained with minimum discomfort or risk to the patient, specific, sensitive, reproducible, objective, quantifiable and economical. Even though historically the term “biomarker” included physical traits or physiological metrics, the term has expanded to molecular biomarkers in the last decades. The term “molecular biomarker” is a broad concept that encompasses a variety of components such as specific cells, proteins, hormones, enzymes, molecules, RNAs, miRNAs, genes and specific mutations, among others.

Although the research in cancer in the last years have been focused on the early diagnosis of the tumors, the developed tools are scarce and insufficient; thus, molecular diagnostic biomarkers with a high sensitivity and specificity are still needed to develop new tools that permit to improve cancer survival over the world.

4.2 Tissue biopsy

Cancers arise owing to the accumulation of molecular alterations in genes that control cell survival, growth, proliferation, and differentiation within the nascent tumor. Thus, it has been a straightforward approach followed by a great number of scientists in the quest of biomarker research to search for those alterations, directly on tumor tissue specimens, to identify biomarkers. Although using tissues as a source of biomarkers present the advantage of that any potential biomarker in the altered tissue will present a higher concentration compared to any other source of

biomarkers, profiling approaches on tissues are subject to some important disadvantages. As tumors tend to be heterogeneous, they usually consist of diverse subpopulations so, there is a chance that some of their characteristics, maybe even the most aggressive subclones, remain undetected with the small amount of tissue obtained by needle biopsies [59, 60]. Moreover, tissue biopsies are usually taken from the primary tumor and reflect its molecular composition at the time of the sample taken, but the molecular profile of tumors evolves dynamically over time[61] so routine monitoring requires the patient to endure pain during multiple biopsy procedures in the course of treatment[62] and sometimes, the mere analysis of the resected primary tumor alone (current standard practice in oncology) may provide misleading information with regard to the characteristics of metastases, the key target for systemic anticancer therapy [59]. Regarding the localization of the tumor, in some cases, the tumor entities are located at remote sites and a needle biopsy can be very difficult and at high risk [59] so accessibility could be a problem.

Standard molecular analysis of proteins and nucleic acids in the tumor tissue biopsy, performed using robust clinical equipment for sequencing, immune profiling, and cell processing, are also time-consuming, expensive, and require extensive technical expertise from clinicians [63]. In addition, assessments of tumor specimens can be restricted by time-related hindrances, including the reliability of molecular tests using years-old archival samples, and the unfeasibility of performing multiple longitudinal tests to follow tumor evolution and thereby expose mechanisms of secondary resistance to treatment [61].

Biomarkers are envisaged to not only provide opportunities for disease early detection and screening, but also for molecular classification, differential diagnosis, prediction of disease progression, patient selection and stratification, prediction of therapeutic response and response monitoring. With strategies in cancer management continuing to evolve, the challenges and demands associated continue

to grow in tandem and that is the reason why advances in cancer research and biomedical technology have enabled development of new source of biomarkers to complement conventional biopsies.

4.3 Liquid biopsy, a new source of biomarkers

Nowadays, research efforts are focused on the discovery of new non-invasive methods for the diagnosis and comprehension of the tumor molecular architecture in real time. In comparison with traditional biopsies, the study of the tumor material present in body fluids can provide valuable information. Liquid biopsies are growing in popularity because of their minimal invasiveness, ease-of-use, and high throughput for personalized analyses [64, 65, 66, 67]. Liquid biopsy provides a promising approach for early diagnosis, therapeutic and prognostic decision making, and monitoring through minimally invasive fluid sample collections rather than a more invasive biopsy [68]. Liquid biopsy also is expected to be lower cost because of the ease of sample collection and its ease of use in the clinic. In all these senses, the field of liquid biopsy has emerged as a great revolution in oncology and is considered "the way" to reach precision medicine [63, 69, 70].

The main tumor circulating biomarkers that have been studied in liquid biopsy for cancer include circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), circulating tumor miRNA, proteins and extracellular vesicles [63, 71, 68]. Blood serum or plasma are the most used clinical fluid samples for the identification of biomarkers since they are collected routinely in the laboratory in a rapid and minimally invasive way [61]. In addition to blood, there are a number of sample types that have been used for liquid biopsy such as saliva [72], urine [73], cerebrospinal fluid (CSF)[74], uterine aspirates [75], pleural effusions [76] or even stool [77]. All of them have been shown to contain tumor-derived genetic material, and this has incremented our expectations to exploit liquid biopsies for diagnostic purposes in the future [61].

Applications of liquid biopsies in oncology have emerged and developed at an incredible rate over the past 5 years. Exploiting liquid biopsy approaches in patient screening could provide a more comprehensive view of tumor characteristics, including aggressiveness and the overall molecular landscape.

Proximal fluid, the fluid that is located close in proximity to the tissue of interest, could be secreted naturally or extracted by various techniques. Proximal fluids have been hypothesized to provide a rich source for biomarker discovery. This hypothesis is supported by the notion that the fluids closest to the site of a malignancy, for example, are likely to have a high local concentration of soluble proteins and protein fragments that are produced by active secretion and shedding from the tissue microenvironment. Discovery of biomarkers may be facilitated in proximal fluids due to the high loco-regional concentration of proteins that otherwise are highly diluted in peripheral circulation.

4.4 Exosomes

4.4.1 Definition

Extracellular vesicles (EVs) are small cell-derived vesicles that are released into the extracellular space by most cell types [78] and they carry several types of cargo molecules, therefore are considered to be crucial for the discovery of biomarkers for clinical use. EVs are phospholipid bilayer-enclosed vesicles that act as key mediators of cell-to-cell communication in either physiological or pathological situations via the horizontal transference of biologically active cargo such as proteins, nucleic acids, enzymes, signaling molecules, mRNA, miRNAs, long non-coding RNAs, lipids, sugars and oncomolecules [61, 79].

Typically, two main categories of EVs are secreted from cells, exosomes and microvesicles (MVs), differing for sizes and biogenesis mechanisms (see Figure 13).

Exosomes are the most studied EVs and constitute a rather homogeneous population of small spherical vesicles of approximately 30 to 100 nm in diameter (shown in Table 14) that are produced by budding of the late endosome or multivesicular bodies (MVBs) [80, 81]. On the other hand, MVs are a heterogeneous population of large vesicles ranging from 100–1000 nm in diameter that shed directly from the cellular plasma membrane [82, 83, 84, 85, 86 87]. In addition to these two classes, apoptotic bodies are large (1000 to 5000 nm in diameter) EVs that are specifically released by cells undergoing apoptosis [88].

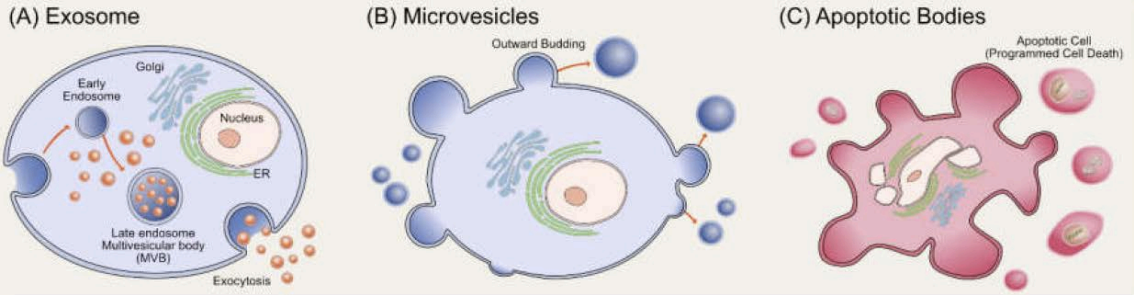


Figure 12. Schematic representation of EVs. Adapted from Gurunathan et al [89].

Feature	Exosomes	Microvesicles	Apoptotic bodies
Size	30-150 nm	50-100 nm	50-5.000 nm
Density in sucrose	1.13-1.19 g/ml	ND	1.16-1.28 g/ml
Appearance by electron microscopy	Cup shape	Irregular shape and electron dense	Heterogeneous
Sedimentation	100,000xg	10,000xg	1,200xg, 10,00xg or 100,000xg
Lipid composition	Enriched in cholesterol, sphingomyelin and ceramide; contain lipid rafts; expose phosphatidylserine	Expose posphatidylserine	ND
Main protein markers	Tetraapanins (CD63, CD9, CD81), Alix and TSG101	Integrins, selectines and CD40 ligand, ARF6	Histones, Annexine V, Caspase 3
Intracellular origin	Endosomes	Plasma membrane	ND

Table 14. Physicochemical characteristics of different types of EVs. ND: not determined. Adapted from Ciardiello et al [90] and Théry et al [91].

4.4.2 Biological functions of exosomes

EVs play a significant role in intercellular communication by serving as a carrier for the transfer of membrane and cytosolic proteins, lipids, and RNA between cells [80] (composition of EVs is resumed in Figure 14).

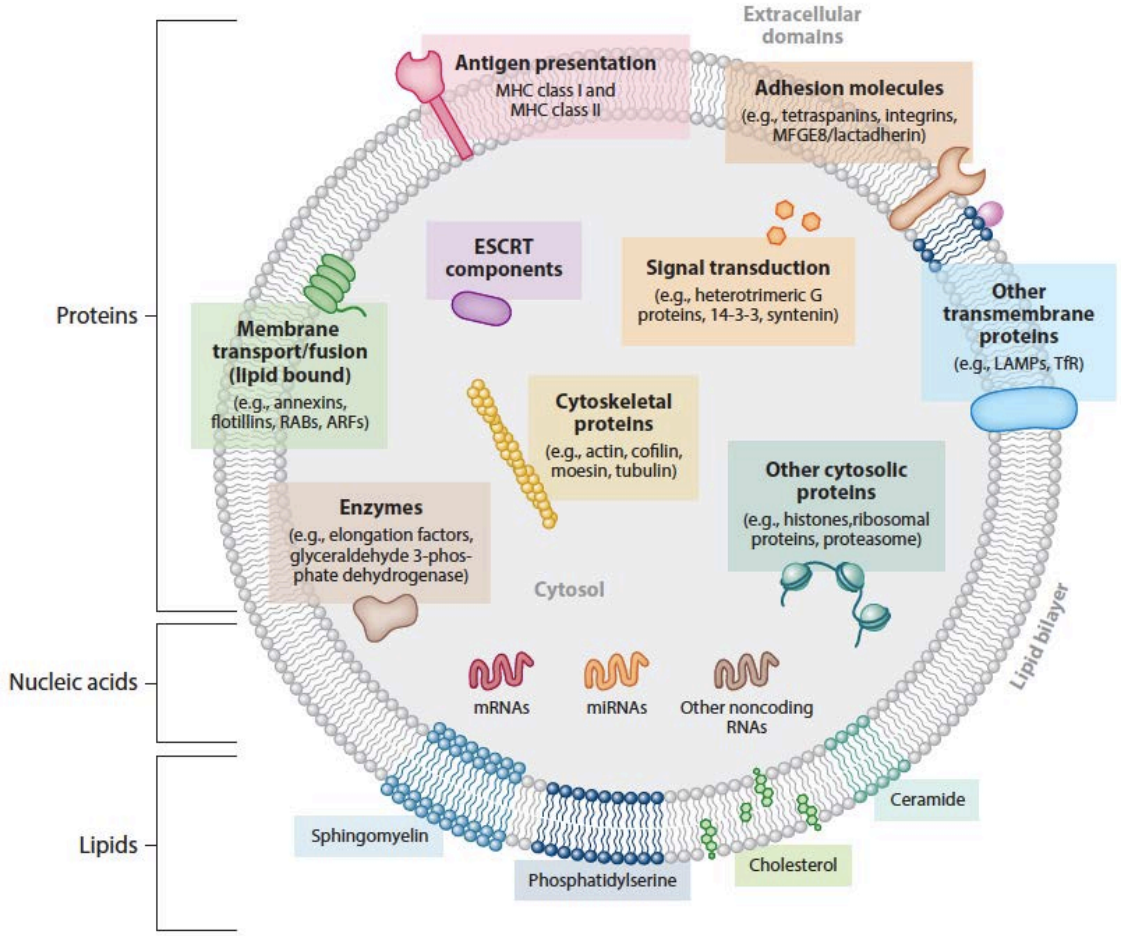


Figure 13. Overall composition of EVs. Schematic representation of the composition (families of proteins, lipids, and nucleic acids) and membrane orientation of EVs. Examples of tetraspanins commonly found in EVs include CD63, CD81, and CD9. Adapted from Colombo et al [81].

Recent studies have shown that exosomes play significant roles in various biological processes, such as angiogenesis, antigen presentation, apoptosis, coagulation, cellular homeostasis, inflammation, and intercellular signaling [92, 93] (see Figure 15).

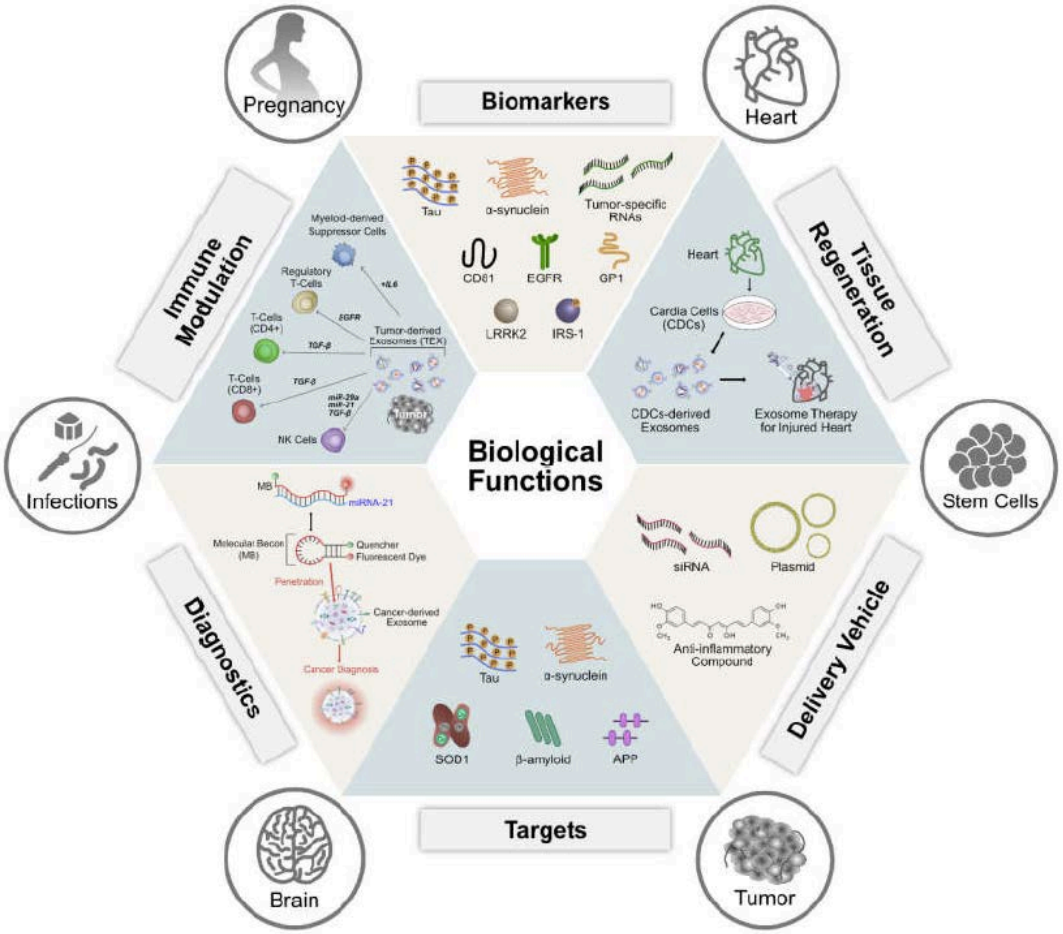


Figure 14. Biological functions of exosomes. Adapted from Gurunathan et al [89].

These roles are affecting physiological but also pathological processes in various diseases, including cancer, neurodegenerative diseases, infections, and autoimmune diseases [94]. A large body of evidence suggests that cancer cells release higher amounts of EVs, which should be involved in processes leading to transformation from non-malignant to malignant phenotypes in the recipient cells [82, 95]. Recent studies reveal that EVs released by cancer cells can affect tumor microenvironment inducing stromal cells to adopt proangiogenic, prometastatic, or immune suppressive phenotype [96]. Moreover, cancer EVs can contribute to cancer progression and remodeling of the extracellular matrix (ECM) by delivering growth factors, adhesion molecules, and metalloproteases [97, 98]. Recently, EVs have also

been described as a participants in the pre-metastatic niche formation altering the behavior of bone marrow-derived progenitors or resident specialized cells [99].

4.4.3 Isolation and characterization methods

EVs have been found and isolated from diverse body fluids, including blood, plasma, urine, saliva, mother milk, semen, CSF, synovial fluid, epididymal fluid, amniotic fluid, malignant and pleural effusions of ascites and bronchoalveolar lavage, among others [100, 101, 102, 103].

Currently, EVs are isolated by a variety of methods that influence amount, type, and purity of the recovered EVs [104, 105] and the specific method used to isolate EVs is critical to the success of the isolation [89]. Each isolation technique presents advantages and disadvantages and the choice of methods should be based on different factors, such as starting material, volume, desired grade of purity, and isolation purpose (research, therapeutic, or diagnostic use). Several conventional methods have been employed to isolate exosomes. These include differential and buoyant density centrifugation, ultrafiltration, size exclusion, precipitation, immunoaffinity separation, and differential centrifugation, being the latter the most commonly used method to isolate EVs [106, 107, 108]. Once isolated, vesicles should be characterized by any combination of the following methods: immunoblotting, transmission electron microscopy (TEM), scanning electron microscopy (SEM), atomic force microscopy (AFM), nanoparticle tracking analysis (NTA), dynamic light scattering (DLS), resistive pulse sensing, enzyme-linked immunosorbent assay (ELISA), flow cytometry, fluorescence-activated cell sorting (FACS), and microfluidics and electrochemical biosensors [109, 106]. This is important in order to understand the exosome purity and to quantify exosomal cargo.

4.4.4 Exosomes as a source of biomarkers

EVs are considered as valuable sources for biomarkers of the pathophysiology of several diseases mainly because they are released by most cell types, representing the cell of origin, and they are present and stable in most body fluids.

In consequence, exosomes are qualified as minimally invasive source of biomarkers for early detection, diagnosis and prognosis of cancer. Moreover, the presence of EVs in biofluid samples (blood, urine, saliva, CSF) is a great opportunity for diagnostic innovation as they can be potential candidates for the so-called "liquid biopsy" (see Figure 16), which is emerging as a powerful method to monitor treatment efficacy, drug resistance, and evolution of disease, avoiding the need for repeated invasive examinations on patients over time [61].

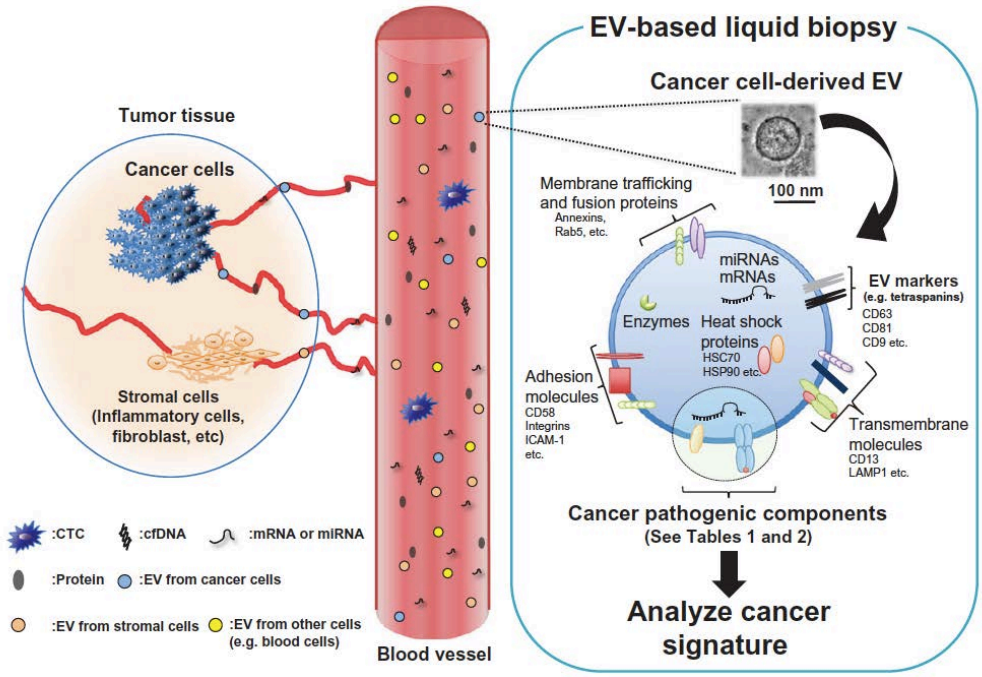


Figure 15. Schematic of EV-based liquid biopsy. Liquid biopsies of tumor components in the blood, including CTC, ctDNA, extracellular RNAs and EVs, can be leveraged to diagnose for tumor. Molecular analysis of these different components can provide a lot of information. Adapted from Yoshioka et al [110].

4.5 Exosomal miRNA as a biomarkers in cancer research

EVs, that can be isolated from liquid biopsy, carry several types of cargo molecules, such as nucleic acids and proteins and are, therefore, considered to be crucial for the discovery of biomarkers for clinical diagnostics. In specific, exosomes loaded with tumor-specific miRNAs that could be considered as potential biomarkers for a variety of diseases, including cancer [89].

miRNAs are highly conserved family of small endogenous non-coding and single-stranded RNAs that are 20–22 nucleotides in length. They negatively regulate gene expression by base pairing to partially complementary sites on target mRNAs, usually in the three primer untranslated region (3'- UTR). Binding of a miRNA to its target mRNA typically leads to translational repression and exonucleolytic mRNA decay, although highly complementary targets can be cleaved endonucleolytically [111]. MiRNAs are involved in crucial biological processes, including development, differentiation, apoptosis and proliferation, through imperfect pairing with target mRNAs of protein-coding genes and the transcriptional or post-transcriptional regulation of their expression. MiRNAs have been proposed to contribute to oncogenesis because they can function either as tumor suppressors or oncogenes [112].

One breakthrough regarding cancer diagnosis using miRNA was the discovery of miRNA in EVs. Valadi et al. showed that mouse and human mast cell-derived EVs contain miRNA. After this report, in 2008, three independent studies demonstrated that miRNAs are released into the circulation and exist there in a remarkably stable form, thereby suggesting that extracellular miRNAs may carry disease specific signatures that could be exploited as non-invasive biomarkers. In particular, miRNAs in body fluids, which are not associated with vesicles, were reported to show differential stability to treatment by RNase A, suggesting that EV-associated miRNAs are preferable as biological specimens for developing diagnostic biomarkers

because of their stability in body fluid [110]. Many studies have attempted to identify EV-associated miRNAs with diagnostic, prognostic or predictive relevance in body fluids from patients with various diseases among cancer [113].

OBJECTIVES

Under the assumption that exosome-like vesicles exist in most of the body fluids and that, in the context of EC, CRC and LC, a high proportion of these vesicles might be released by tumor cells into proximal fluids, we hypothesize that the miRNA profile study on exosome-like vesicles from peritoneal lavage of EC and CRC patients and pleural lavage of LC patients will provide a set of biomarkers useful to provide information of those diseases for the clinical management of the patients.

The main objective of this work is to evaluate the potential of miRNA signatures in exosomes-like vesicles derived from peritoneal and pleural lavage to provide relevant information for EC, CRC and LC. This might help to improve the detection and management of EC, CRC and LC patients and overcome the mortality rate of EC, CCR and LC. Towards this end, this thesis has focused on three very specific objectives:

1. **Identification of EV-associated miRNAs biomarkers of EC in peritoneal lavage.**
   - Establishment and optimization of a suitable protocol for the isolation of EV-associated miRNA from peritoneal lavage and ascitic fluid from EC patients and control patients, respectively.
   - Characterization of EVs from peritoneal lavage and ascitic fluid.
   - Analysis of EV-associated miRNA profile by Taqman Open Array technology.
   - Differential expression analysis to identify potential miRNA biomarkers.
   - Bioinformatic study on the altered biological processes and molecular functions related to the potential biomarkers.

2. **Identification of EV-associated miRNAs biomarkers of CRC in peritoneal lavage.**
   - Establishment and optimization of a suitable protocol for the isolation of EV-associated miRNA from peritoneal lavage and ascitic fluid from CRC patients and control patients, respectively.
   - Characterization of EVs from peritoneal lavage and ascitic fluid.
   - Analysis of EV-associated miRNA profile by Taqman Open Array technology.
   - Differential expression analysis to identify potential miRNA biomarkers.
   - Bioinformatic study on the altered biological processes and molecular functions related to the potential biomarkers.

3. **Identification of EV-associated miRNAs biomarkers of LC in pleural lavage.**
   - Establishment and optimization of a suitable protocol for the isolation of EV-associated miRNA from pleural lavage and pleural fluid from LC patients and control patients, respectively.
   - Characterization of EVs from pleural lavage and pleural fluid.
   - Analysis of EV-associated miRNA profile by Taqman Open Array technology.
   - Differential expression analysis to identify potential miRNA biomarkers.
   - Bioinformatic study on the altered biological processes and molecular functions related to the potential biomarkers.

RESULTS

The results generated during this thesis work are divided into 3 chapters, each one of them corresponding to a paper. Chapter I and Chapter II are published papers and contain the original manuscript with the supplementary information and the references included at the end of the chapter. Chapter III is a manuscript under revision in a journal with impact factor and it also includes supplementary information and references attached at the end of the chapter.

CHAPTER I

EV-Associated miRNAs from Peritoneal Lavage are a Source of Biomarkers in Endometrial Cancer

Berta Roman-Canal, Cristian Pablo Moiola, Sònia Gatius, Sarah Bonnin, Maria Ruiz-Miró, Esperanza González, Xavier González-Tallada, Ivanna Llordella, Isabel Hernández, José M. Porcel, Antonio Gil-Moreno, Juan M. Falcón-Pérez, Julia Ponomarenko, Xavier Matias-Guiu and Eva Colas

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Article

EV-Associated miRNAs from Peritoneal Lavage are a Source of Biomarkers in Endometrial Cancer

Berta Roman-Canal [1,2,3,4], Cristian Pablo Moiola [1,3], Sònia Gatius [1], Sarah Bonnin [5], Maria Ruiz-Miró [1], Esperanza González [6], Xavier González-Tallada [1], Ivanna Llordella [1], Isabel Hernández [1], José M. Porcel [7], Antonio Gil-Moreno [3,4,8], Juan M. Falcón-Pérez [6,9], Julia Ponomarenko [5,10], Xavier Matias-Guiu [1,2,*] and Eva Colas [3,*]

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Abstract: Endometrial cancer (EC) is the sixth most common cancer in women worldwide and is responsible for more than 89,000 deaths every year. Mortality is associated with presence of poor prognostic factors at diagnosis, i.e., diagnosis at an advanced stage, with a high grade and/or an aggressive histology. Development of novel approaches that would permit us to improve the clinical management of EC patients is an unmet need. In this study, we investigate a novel approach to identify highly sensitive and specific biomarkers of EC using extracellular vesicles (EVs) isolated from the peritoneal lavage of EC patients. EVs of peritoneal lavages of 25 EC patients were isolated and their miRNA content was compared with miRNAs of EVs isolated from the ascitic fluid of 25 control patients. Expression of the EV-associated miRNAs was measured using the Taqman OpenArray technology that allowed us to detect 371 miRNAs. The analysis showed that 114 miRNAs were significantly dysregulated in EC patients, among which eight miRNAs, miRNA-383-5p, miRNA-10b-5p, miRNA-34c-3p, miRNA-449b-5p, miRNA-34c-5p, miRNA-200b-3p, miRNA-2110, and miRNA-34b-3p, demonstrated a classification performance at area under the receiver operating characteristic curve (AUC) values above 0.9. This finding opens an avenue for the use of EV-associated miRNAs of peritoneal lavages as an untapped source of biomarkers for EC.

Keywords: endometrial cancer; uterine cancer; exosomes; biomarkers; miRNAs; ascitic fluid; peritoneal lavage; liquid biopsy; extracellular vesicles; microRNAs

1. Introduction

Endometrial cancer (EC) is the sixth most common cancer in women worldwide and is responsible for more than 89,000 deaths every year [1]. EC is predominantly a disease that afflicts postmenopausal women, occurs in women older than 50 years in more than 90% of cases, and is detected at a mean age of 65 [2]. Approximately 10% of cases are diagnosed in premenopausal women, 5% of whom are younger than 40 years. Mortality is associated with presence of poor prognostic factors at diagnosis, i.e., diagnosis at an advanced stage, with a high grade and/or an aggressive histology. Patients presenting any of those features are at increased risk of recurrence, and, for them, therapeutic options are limited. Although most ECs are diagnosed early, up to 10% of tumors are diagnosed at a late stage, where the five-year survival drops to 16% compared to 95% in women diagnosed at an early stage [3]. Regarding histology, 80% of EC patients are diagnosed with an endometrioid histology with the average 5-year survival of 75%. Nonetheless, the 20% of patients diagnosed with a non-endometrioid histology account for 47% of EC-related deaths. Grade 3 endometrioid tumors are diagnosed in 15% of all EC patients, although theses tumors are responsible for 27% of EC-related deaths.

The cornerstone treatment of EC is surgery, which is mostly standardized through all hospitals with slight variations. The national comprehensive cancer network (NCCN) recommends both surgical and pathological staging with total hysterectomy, bilateral salpingo-oophorectomy, and peritoneal cytology. Although lymphadenectomy still remains the most reliable way of avowing downstaging and correctly identifying patients who require adjuvant chemotherapy or radiotherapy, recent data have questioned its role in early stage EC due to the high variability in node involvement and the frequently associated comorbidities. Lymphadenectomy might not be performed in early stage EC patients in some centers. Moreover, fertility-sparing surgery (FSS) in reproductive-age patients affected by endometrial cancer has attracted attention in the last decade because the consequences of an approach that is too radical may have a severe impact on a patient's quality of life and psychological well-being [4].

The new era of molecular advances has fostered biomarker research, although the identification of useful biomarkers in liquid biopsies remains a challenge. Among all serum biomarkers, the human epididymis protein 4 (HE4) has been one of the most investigated in EC. HE4 was found to be sufficiently specific but poorly sensitive in patients with EC. The diagnostic performance of HE4 appears to be better than that of the cancer antigen 125 (CA125) in diagnosing EC at an early stage, but its real value and efficacy for management of EC have not been clearly demonstrated in clinical practice [5]. Hence, a clinical challenge in EC is the development of novel molecular approaches to liquid biopsies that permit early diagnosis and recurrence control.

MicroRNAs (miRNAs) are small, non-coding RNA molecules of about 22 nucleotides in length [6] that regulate gene expression at the post-transcriptional level by inhibiting protein translation or destabilizing target transcripts via binding to the 3'-untranslated region (3'UTR), resulting in transcriptional repression or mRNA degradation upon dicer cleavage [7]. miRNAs have been found to play a critical role in almost every physiological process, including differentiation, proliferation, and apoptosis. They have also been described as oncogenes or tumor suppressors in some tumors [8], including EC [6]. Although miRNAs are detected intracellularly, they pass into the extracellular space and can be detected in a broad variety of bodily fluids, either freely in circulation or contained in extracellular vesicles (EVs) [9].

There are different types of EVs, and their size ranges from 20 to 200 nm. The largest vesicles, i.e., microvesicles, are released directly from the budding of the plasma membrane. The smallest vesicles, i.e., exosomes, are formed within intracellular multivesicular bodies and released by their fusion with the cellular membrane. Their function is to mediate intercellular communication, influencing the recipient cell's behavior. Importantly, EVs have attracted the interest of the scientific community as a source of biomarkers, mainly because they carry a broad range of bioactive material (proteins, metabolites, RNA, miRNA, etc.) that is well-protected by the lipid bilayer membrane of EVs, even if they are extracted from circulating or proximal bodily fluids or frozen before any experimental study [10].

Herein, we investigate the use of EVs isolated from the peritoneal lavage, a proximal fluid of EC, as a source of potential EC biomarkers. The peritoneal lavage, at surgery, just before starting the manipulation of the uterus, was used for staging purposes according to the old International Federation of Gynecology and Obstretrics (FIGO) staging rules. In several centers, peritoneal washing is still performed because of the prognostic information that the presence of cancer cells provides by cytologic examination. However, this fluid has not been used for molecular analysis. In this study, we conducted miRNA profiling of EVs isolated from the peritoneal lavages of 25 EC patients and the ascitic fluids of 25 non-cancer patients using the TaqMan OpenArray Human MicroRNA Panel. We identified the most relevant individual miRNAs related to EC and characterized the biological and molecular landscape of the EC milieu. The study was conceived as a proof of concept investigation to demonstrate the feasibility of using the peritoneal lavage as a source of EV-associated miRNA biomarkers of EC.

2. Results

We analyzed the miRNA profile of EVs isolated from the ascitic fluids of 25 control patients and the peritoneal lavages of 25 EC patients. Figure 1 illustrates the workflow that was followed in this study.

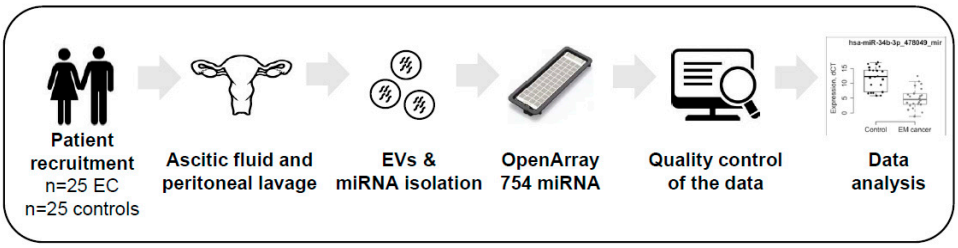

Figure 1. Workflow of the study design. Abbreviations: EC, Endometrial Cancer; EVs, Extracellular vesicles.

The quality of EVs isolated from the ascitic fluids and peritoneal lavages was measured by size distribution and concentration by Nanoparticle Tracking Analysis. The analysis demonstrated that we analyzed a population that was mostly comprised of small EVs but that also contained a low number of microvesicles. The EVs isolated from EC and non-EC patients did not differ in concentration and size (Supplementary Figure S1). miRNAs were extracted from all EVs and they were used for a systematic miRNA expression analysis using the Taqman OpenArray technology. We detected 371 out of the 754 miRNAs (49.2%) present in the OpenArray. Probes that had a Cycle threshold (Ct) value of 40 in all samples and samples in which more than 80% of the probes had a Ct value greater than 40 were removed, resulting in a study that contained a total of 355 miRNAs from 22 control and 22 EC patients (Table 1).

Table 1. Clinicopathological characteristics of patients.

Clinical Parameters	Endometrial Cancer	Control
Age		
Median	61.4	65.5
Minimum	46	52
Maximum	78	90
Gender		
Female	22	4
Male	-	18
Pathology		
Endometrial Cancer		-
EEC	19	-
NEEC	3	-
Hepatic cirrhoses	-	20
Other	-	22

Clinical characteristics of the final cohort of patients included in the study after data normalization.

The differential expression analysis between cancer and control cases yielded a list of 114 miRNAs that were significantly dysregulated (adj. $p < 0.05$ and abs(logFC) > = 1). Among those, 96 miRNA were found to be downregulated and 18 miRNA were upregulated in EC patients (Table 2). To evaluate whether these miRNAs can be used as biomarkers, we performed a predictive analysis using the logistic modeling. Eight miRNAs demonstrated predictive performance with area under the receiver operating characteristic curve (AUC) values above 0.90, including miRNA-383-5p, miRNA-10b-5p, miRNA-34c-3p, miRNA-449b-5p, miRNA-34c-5p, miRNA-200b-3p, miRNA-2110, and miRNA-34b-3p (Table 2, rows in bold; Figure 2). All eight miRNAs were significantly downregulated in EC patients (from 3.75-fold to 12.18-fold in the log2_scale).

Table 2. List of MicroRNA (miRNA) transcripts displaying a significant differential expression in patients with EC compared to control patients.

miRNA	logFC	*p*-Value	adj.*p*-Value	AUC	AUC_95%CI_Lower	AUC_95%CI_Upper	Accuracy	Sensitivity	Specificity
hsa-miR-383-5p_478079_mir	**−7.56**	**2.86E-14**	**1.02E-11**	**0.961**	**0.938**	**0.985**	**0.884**	**0.876**	**0.892**
hsa-miR-10b-5p_478494_mir	**−3.75**	**2.62E-11**	**3.10E-09**	**0.958**	**0.93**	**0.987**	**0.897**	**0.864**	**0.93**
hsa-miR-34c-3p_478051_mir	**−10.56**	**9.57E-10**	**6.79E-08**	**0.948**	**0.925**	**0.97**	**0.843**	**0.893**	**0.794**
hsa-miR-449b-5p_479528_mir	**−12.18**	**2.58E-11**	**3.10E-09**	**0.927**	**0.896**	**0.959**	**0.864**	**0.896**	**0.83**
hsa-miR-34c-5p_478052_mir	**−5.97**	**5.60E-10**	**4.97E-08**	**0.924**	**0.891**	**0.957**	**0.845**	**0.86**	**0.83**
hsa-miR-200b-3p_477963_mir	**−6.10**	**2.73E-06**	**6.91E-05**	**0.917**	**0.888**	**0.946**	**0.846**	**0.824**	**0.869**
hsa-miR-2110_477971_mir	**−4.57**	**7.27E-07**	**2.58E-05**	**0.906**	**0.875**	**0.938**	**0.802**	**0.748**	**0.855**
hsa-miR-34b-3p_478049_mir	**−6.73**	**4.17E-07**	**2.12E-05**	**0.903**	**0.874**	**0.932**	**0.752**	**0.719**	**0.786**
hsa-miR-200c-3p_478351_mir	−10.29	3.42E-09	2.02E-07	0.897	0.863	0.931	0.799	0.744	0.853
hsa-miR-150-5p_477918_mir	2.89	9.74E-06	2.26E-04	0.892	0.856	0.928	0.861	0.908	0.814
hsa-miR-1180-3p_477869_mir	−4.62	9.40E-07	3.04E-05	0.89	0.854	0.926	0.791	0.738	0.844
hsa-miR-29c-5p_478005_mir	−2.39	1.35E-06	3.70E-05	0.883	0.849	0.918	0.773	0.773	0.774
hsa-miR-190a-5p_478358_mir	−2.51	1.42E-03	9.18E-03	0.871	0.831	0.911	0.816	0.813	0.82
hsa-miR-708-5p_478197_mir	−5.51	1.19E-06	3.52E-05	0.865	0.826	0.903	0.77	0.684	0.856
hsa-miR-218-5p_477977_mir	−6.70	1.27E-05	2.59E-04	0.859	0.816	0.901	0.715	0.53	0.9
hsa-miR-99a-3p_479224_mir	−3.23	4.78E-05	7.78E-04	0.858	0.818	0.897	0.732	0.733	0.73
hsa-miR-196b-5p_478585_mir	−6.63	5.15E-07	2.29E-05	0.85	0.805	0.894	0.766	0.624	0.908
hsa-miR-142-5p_477911_mir	8.86	1.89E-05	3.35E-04	0.849	0.808	0.889	0.748	0.898	0.599
hsa-miR-193a-3p_478306_mir	−2.49	1.31E-03	8.93E-03	0.849	0.807	0.891	0.789	0.853	0.725
hsa-miR-193a-5p_477954_mir	−3.10	1.02E-05	2.26E-04	0.848	0.808	0.888	0.791	0.78	0.802
hsa-miR-181a-5p_477857_mir	−1.71	1.01E-04	1.49E-03	0.847	0.807	0.887	0.755	0.831	0.678
hsa-miR-125b-5p_477885_mir	−1.86	5.22E-05	8.06E-04	0.845	0.803	0.887	0.744	0.784	0.704
hsa-miR-429_477849_mir	−3.07	2.17E-04	2.65E-03	0.843	0.797	0.89	0.792	0.817	0.767
hsa-miR-30a-3p_478273_mir	−1.72	4.82E-05	7.78E-04	0.841	0.803	0.88	0.698	0.738	0.657
hsa-miR-196a-5p_478230_mir	−6.80	1.89E-05	3.35E-04	0.837	0.801	0.873	0.743	0.803	0.682
hsa-miR-34b-5p_478050_mir	−7.34	6.87E-07	2.58E-05	0.835	0.787	0.884	0.795	0.855	0.735
hsa-miR-769-5p_478203_mir	−2.81	1.60E-03	1.01E-02	0.833	0.788	0.878	0.768	0.724	0.812
hsa-miR-20a-5p_478586_mir	−1.74	2.60E-04	2.88E-03	0.832	0.789	0.875	0.727	0.738	0.716
hsa-miR-320a_478594_mir	−8.30	8.68E-04	6.84E-03	0.831	0.796	0.867	0.698	0.59	0.806
hsa-miR-30d-5p_478606_mir	−2.36	1.01E-02	3.59E-02	0.827	0.785	0.869	0.728	0.746	0.709
hsa-miR-409-3p_478084_mir	3.24	2.24E-04	2.65E-03	0.822	0.777	0.868	0.779	0.872	0.687
hsa-miR-30b-5p_478007_mir	−2.57	1.65E-03	1.01E-02	0.82	0.777	0.863	0.714	0.736	0.693
hsa-miR-598-3p_478172_mir	−1.84	3.68E-04	3.53E-03	0.82	0.778	0.861	0.723	0.677	0.77
hsa-miR-369-3p_478067_mir	4.94	1.32E-04	1.81E-03	0.816	0.772	0.86	0.709	0.895	0.523
hsa-miR-187-3p_477941_mir	−6.40	1.10E-04	1.56E-03	0.815	0.768	0.861	0.749	0.638	0.86
hsa-miR-210-3p_477970_mir	−1.85	1.37E-04	1.81E-03	0.813	0.768	0.859	0.699	0.713	0.686
hsa-miR-29c-3p_479229_mir	−2.84	1.63E-03	1.01E-02	0.812	0.773	0.851	0.742	0.749	0.735
hsa-miR-885-5p_478207_mir	−3.98	3.50E-04	3.45E-03	0.812	0.768	0.857	0.713	0.741	0.686
hsa-miR-142-3p_477910_mir	3.77	2.85E-04	2.98E-03	0.81	0.764	0.857	0.71	0.775	0.645

Table 2. *Cont.*

miRNA	logFC	*p*-Value	adj.*p*-Value	AUC	AUC_95%CI_Lower	AUC_95%CI_Upper	Accuracy	Sensitivity	Specificity
hsa-miR-342-3p_478043_mir	2.17	5.34E-03	2.44E-02	0.808	0.763	0.854	0.721	0.739	0.703
hsa-miR-17-3p_477932_mir	−6.78	2.76E-04	2.97E-03	0.806	0.762	0.85	0.739	0.826	0.652
hsa-miR-92a-3p_477827_mir	−1.49	5.35E-04	4.87E-03	0.805	0.763	0.848	0.711	0.796	0.626
hsa-miR-29a-5p_478002_mir	−6.21	2.46E-03	1.34E-02	0.803	0.762	0.843	0.681	0.802	0.561
hsa-miR-30e-3p_478388_mir	−4.45	6.43E-03	2.78E-02	0.796	0.749	0.844	0.633	0.426	0.839
hsa-miR-125a-5p_477884_mir	−1.39	1.17E-03	8.33E-03	0.794	0.749	0.839	0.746	0.768	0.723
hsa-miR-130a-3p_477851_mir	−1.76	4.73E-04	4.42E-03	0.793	0.744	0.842	0.723	0.836	0.61
hsa-miR-485-3p_478125_mir	2.34	6.14E-03	2.72E-02	0.793	0.746	0.841	0.694	0.692	0.698
hsa-miR-223-3p_477983_mir	1.80	6.62E-04	5.73E-03	0.79	0.744	0.837	0.714	0.756	0.672
hsa-miR-21-5p_477975_mir	1.63	7.79E-04	6.55E-03	0.787	0.738	0.836	0.714	0.706	0.721
hsa-miR-194-5p_477956_mir	−1.29	7.80E-03	3.18E-02	0.785	0.735	0.835	0.749	0.746	0.752
hsa-miR-29a-3p_478587_mir	−2.40	8.07E-03	3.22E-02	0.784	0.736	0.833	0.663	0.746	0.581
hsa-miR-887-3p_479189_mir	−5.02	2.54E-04	2.88E-03	0.784	0.736	0.832	0.652	0.46	0.843
hsa-miR-135a-5p_478581_mir	−5.63	7.93E-04	6.55E-03	0.775	0.727	0.823	0.698	0.663	0.732
hsa-miR-31-5p_478015_mir	−2.16	8.14E-04	6.56E-03	0.775	0.727	0.823	0.671	0.769	0.573
hsa-miR-29b-2-5p_478003_mir	−2.82	8.96E-04	6.92E-03	0.773	0.723	0.824	0.671	0.688	0.654
hsa-miR-26b-5p_478418_mir	−1.47	1.01E-03	7.45E-03	0.771	0.722	0.82	0.669	0.822	0.516
hsa-miR-656-3p_479137_mir	4.06	1.73E-03	1.04E-02	0.771	0.72	0.822	0.693	0.7	0.686
hsa-miR-33b-5p_478479_mir	−4.09	1.48E-02	4.82E-02	0.767	0.718	0.816	0.64	0.591	0.69
hsa-miR-141-3p_478501_mir	−8.97	1.31E-05	2.59E-04	0.765	0.715	0.815	0.718	0.847	0.589
hsa-miR-423-3p_478327_mir	−1.80	3.02E-04	3.07E-03	0.765	0.714	0.816	0.679	0.67	0.687
hsa-miR-28-3p_477999_mir	−2.33	2.24E-03	1.28E-02	0.764	0.714	0.813	0.658	0.621	0.695
hsa-miR-29b-3p_478369_mir	−2.44	6.09E-04	5.40E-03	0.761	0.715	0.808	0.684	0.63	0.737
hsa-let-7b-5p_478576_mir	−1.43	1.22E-03	8.51E-03	0.76	0.715	0.806	0.624	0.648	0.6
hsa-miR-551b-3p_478159_mir	−4.13	2.14E-03	1.25E-02	0.76	0.711	0.809	0.671	0.751	0.591
hsa-miR-154-3p_478725_mir	3.28	2.78E-03	1.47E-02	0.759	0.704	0.814	0.673	0.738	0.608
hsa-miR-18a-3p_477944_mir	−2.84	7.96E-03	3.21E-02	0.759	0.707	0.81	0.67	0.696	0.644
hsa-miR-26a-5p_477995_mir	−1.33	6.71E-03	2.87E-02	0.759	0.709	0.809	0.685	0.622	0.748
hsa-miR-181d-5p_479517_mir	−5.43	4.75E-03	2.31E-02	0.757	0.709	0.805	0.654	0.524	0.784
hsa-miR-151a-3p_477919_mir	−1.65	1.05E-03	7.59E-03	0.756	0.701	0.811	0.731	0.794	0.667
hsa-miR-449a_478561_mir	−5.77	2.18E-04	2.65E-03	0.755	0.701	0.808	0.712	0.812	0.611
hsa-miR-219a-5p_477980_mir	−3.58	2.31E-03	1.30E-02	0.752	0.704	0.8	0.686	0.708	0.664
hsa-miR-584-5p_478167_mir	−5.62	2.55E-03	1.37E-02	0.752	0.703	0.801	0.662	0.778	0.545
hsa-miR-20b-5p_477804_mir	−4.91	3.96E-03	2.04E-02	0.747	0.698	0.796	0.674	0.784	0.563
hsa-miR-24-3p_477992_mir	−1.30	5.35E-03	2.44E-02	0.747	0.696	0.798	0.665	0.688	0.642
hsa-miR-33a-5p_478347_mir	−3.72	2.83E-03	1.48E-02	0.742	0.689	0.795	0.672	0.586	0.757
hsa-let-7e-5p_478579_mir	−6.66	4.07E-03	2.06E-02	0.741	0.685	0.797	0.709	0.794	0.623
hsa-miR-543_478155_mir	2.69	1.40E-03	9.18E-03	0.741	0.687	0.795	0.685	0.544	0.826
hsa-miR-126-5p_477888_mir	5.31	1.01E-03	7.45E-03	0.74	0.686	0.795	0.723	0.826	0.62
hsa-miR-139-5p_478312_mir	−3.63	5.34E-03	2.44E-02	0.738	0.69	0.787	0.641	0.433	0.849
hsa-miR-652-3p_478189_mir	−1.13	1.11E-02	3.75E-02	0.737	0.687	0.786	0.613	0.621	0.605
hsa-miR-200a-3p_478490_mir	−4.89	6.25E-03	2.74E-02	0.735	0.682	0.788	0.709	0.79	0.629
hsa-miR-21-3p_477973_mir	2.34	8.87E-03	3.42E-02	0.735	0.679	0.791	0.689	0.738	0.641

Table 2. *Cont.*

miRNA	logFC	*p*-Value	adj.*p*-Value	AUC	AUC_95%CI_Lower	AUC_95%CI_Upper	Accuracy	Sensitivity	Specificity
hsa-miR-181c-5p_477934_mir	−1.86	1.08E-02	3.73E-02	0.734	0.68	0.789	0.678	0.659	0.698
hsa-miR-361-5p_478056_mir	−1.20	4.86E-03	2.31E-02	0.727	0.677	0.777	0.688	0.75	0.626
hsa-miR-296-5p_477836_mir	−4.15	4.87E-03	2.31E-02	0.726	0.673	0.778	0.631	0.536	0.726
hsa-miR-1271-5p_478674_mir	−4.54	1.01E-02	3.59E-02	0.725	0.674	0.776	0.643	0.783	0.502
hsa-miR-125b-2-3p_478666_mir	−3.58	9.83E-03	3.58E-02	0.724	0.672	0.776	0.637	0.761	0.514
hsa-miR-130b-3p_477840_mir	−4.14	4.77E-03	2.31E-02	0.723	0.673	0.774	0.684	0.66	0.707
hsa-miR-545-3p_479002_mir	−3.15	1.06E-02	3.69E-02	0.723	0.67	0.775	0.648	0.526	0.77
hsa-miR-331-3p_478323_mir	−5.14	7.33E-03	3.10E-02	0.722	0.669	0.775	0.673	0.753	0.594
hsa-miR-128-3p_477892_mir	−1.40	2.39E-03	1.33E-02	0.72	0.667	0.772	0.642	0.647	0.638
hsa-miR-148b-3p_477824_mir	−1.40	7.60E-03	3.17E-02	0.72	0.662	0.778	0.663	0.746	0.58
hsa-miR-30a-5p_479448_mir	−5.80	1.43E-02	4.70E-02	0.716	0.658	0.774	0.654	0.716	0.592
hsa-miR-222-3p_477982_mir	−1.46	1.25E-02	4.20E-02	0.712	0.655	0.77	0.653	0.645	0.661
hsa-miR-451a_478107_mir	−4.53	1.40E-03	9.18E-03	0.711	0.654	0.768	0.61	0.613	0.606
hsa-miR-203a-3p_478316_mir	−4.92	9.36E-03	3.50E-02	0.708	0.653	0.764	0.664	0.804	0.524
hsa-miR-758-3p_479166_mir	3.56	9.37E-03	3.50E-02	0.705	0.653	0.756	0.652	0.692	0.612
hsa-miR-452-5p_478109_mir	−2.16	1.11E-02	3.75E-02	0.703	0.644	0.762	0.598	0.571	0.626
hsa-miR-505-3p_478145_mir	−1.91	6.03E-03	2.71E-02	0.703	0.647	0.759	0.604	0.553	0.655
hsa-miR-504-5p_478144_mir	−2.25	1.06E-02	3.69E-02	0.701	0.647	0.756	0.624	0.58	0.667
hsa-miR-182-5p_477935_mir	−5.24	9.84E-03	3.58E-02	0.7	0.639	0.761	0.686	0.683	0.688
hsa-miR-551a_478158_mir	−4.09	1.58E-02	4.93E-02	0.7	0.644	0.756	0.639	0.772	0.507
hsa-miR-101-3p_477863_mir	−2.79	4.16E-03	2.08E-02	0.699	0.645	0.752	0.626	0.417	0.835
hsa-miR-31-3p_478012_mir	−3.59	8.29E-03	3.27E-02	0.699	0.647	0.751	0.675	0.718	0.632
hsa-miR-140-3p_477908_mir	−2.87	1.56E-02	4.90E-02	0.697	0.642	0.753	0.622	0.439	0.805
hsa-miR-214-5p_478768_mir	−3.30	8.46E-03	3.30E-02	0.696	0.641	0.751	0.649	0.707	0.591
hsa-miR-199b-5p_478486_mir	−4.83	1.49E-02	4.82E-02	0.695	0.646	0.744	0.623	0.631	0.614
hsa-miR-337-5p_478036_mir	3.17	1.27E-02	4.22E-02	0.688	0.631	0.745	0.711	0.9	0.523
hsa-miR-154-5p_477925_mir	2.07	9.88E-03	3.58E-02	0.687	0.631	0.742	0.657	0.773	0.541
hsa-miR-582-5p_478166_mir	−3.50	1.52E-02	4.83E-02	0.681	0.618	0.743	0.644	0.702	0.587
hsa-miR-126-3p_477887_mir	5.32	1.82E-03	1.08E-02	0.678	0.616	0.739	0.738	0.832	0.643
hsa-miR-143-3p_477912_mir	−1.54	9.06E-03	3.46E-02	0.674	0.616	0.731	0.677	0.745	0.608
hsa-miR-324-5p_478024_mir	−1.34	1.52E-02	4.83E-02	0.651	0.588	0.714	0.572	0.603	0.542
hsa-miR-145-5p_477916_mir	−1.70	7.80E-03	3.18E-02	0.649	0.591	0.708	0.646	0.723	0.57

Log fold-change expression, *p*-value, adjusted *p*-value, area under the receiver operating characteristic curve (AUC) values, accuracy, sensitivity, specificity, and 95% confidence intervals of the 114 dysregulated miRNAs.

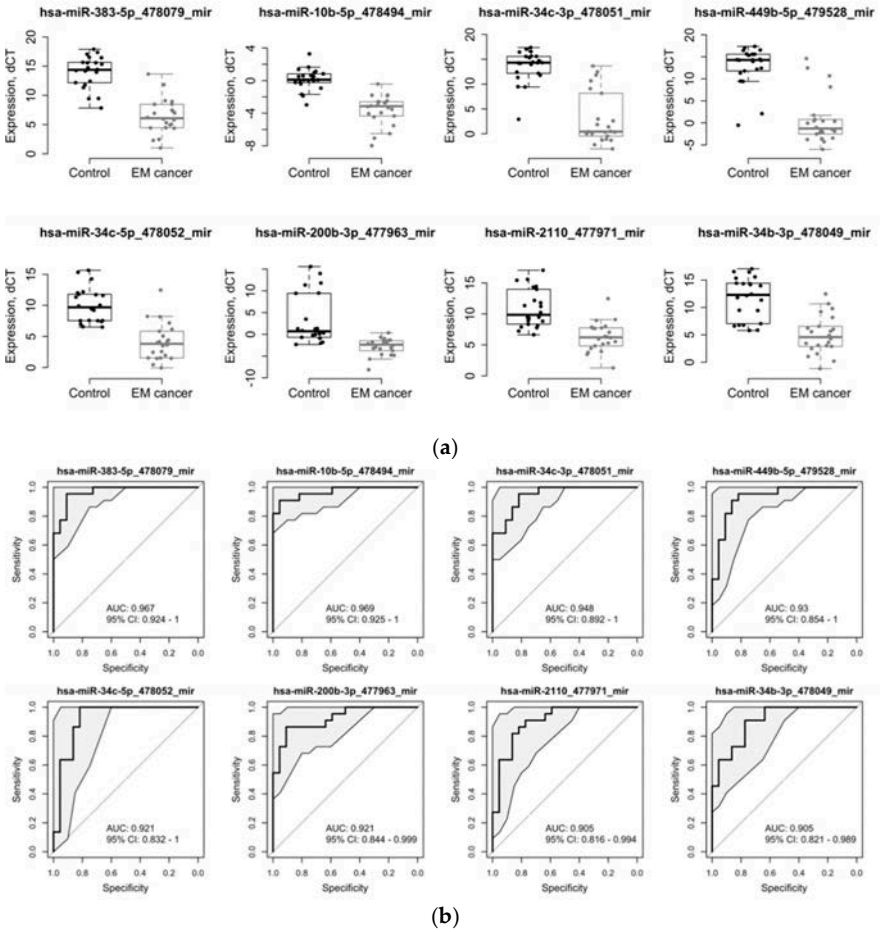

Figure 2. Diagnostic performance of the top eight differentially expressed miRNAs: (**a**) Relative dCT values of the top differentially expressed miRNAs in patients with EC (n = 22) compared to control patients (n = 22), ** $p < 0.05$; (**b**) receiver operating characteristic (ROC) curves and AUC scores for the top eight differentially expressed miRNAs.

To further understand the milieu generated by EVs in the context of EC, we performed a bioinformatics study to first identify the predicted transcripts that are regulated by all of the differentially expressed miRNAs and then to assess the biological processes and molecular functions that they participate in. A total of 8074 transcripts were found to be regulated by the 114 differentially expressed miRNAs. To comprehensively integrate the properties of all target transcripts, they were classified with the Gene Ontology (GO) terms shown in Figure 3A,B.

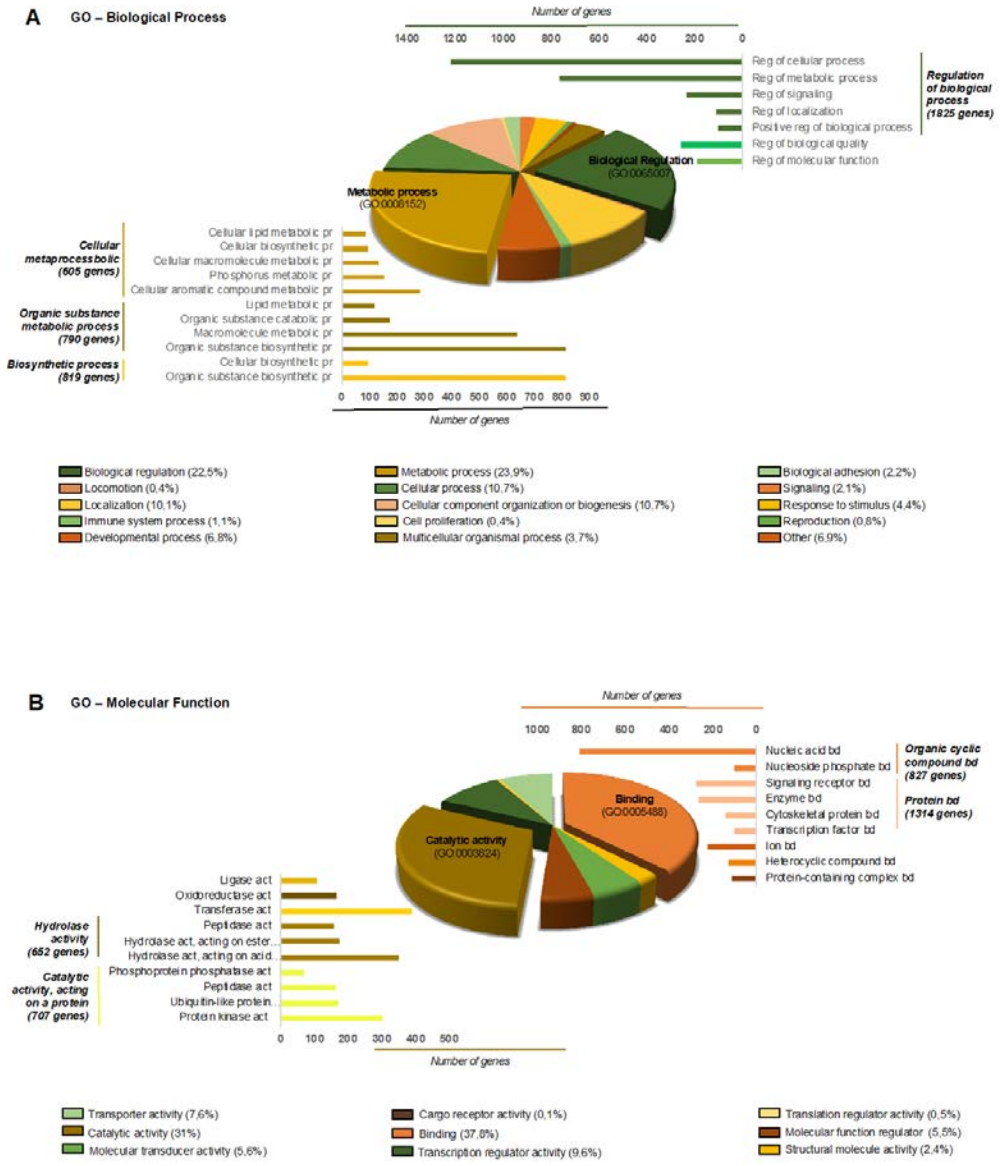

Figure 3. Gene Ontology (GO) analysis of the predicted proteins regulated by miRNA from EVs of EC and control patients: (**A**) Biological process GO analysis of predicted proteins regulated by differentially expressed miRNA from the ELV of EC and control patients. A total of 8074 genes were predicted to be modulated by 114 miRNA and were included in at least one of the GO Biological process categories as indicated in the pie chart. Others include the following categories that have less than 0.4% of representation: growth; multi-organism process; biological phase; rhythmic process; pigmentation; nitrogen utilization. (**B**) Molecular Function GO analysis of predicted proteins regulated by differentially expressed miRNA from the ELV of EC and control patients. A total of 8074 genes were predicted to be modulated by 115 miRNA and were included in at least one of the GO Molecular Function categories as indicated in the pie chart. Abbreviations: Reg, Regulation; Pr, Process; Bd, Binding; Act, Activity.

3. Discussion

In this study, we investigated for the first time the miRNA content of EVs isolated from peritoneal lavages and ascitic fluids of EC and control patients, respectively. Our study shows that the EV-associated miRNAs can be consistently extracted from those proximal bodily fluids and that the miRNA expression profiles can indicate and represent the status of EC patients. The EV-associated miRNAs were analyzed using the Taqman OpenArray technology. The differential expression analysis yielded 114 miRNAs that were significantly dysregulated in EC patients.

An abundance of scientific research has been published regarding the role of miRNAs in EC [11]. Torres et al. published the first study focused on miRNA expression both in tissue and plasma samples of EC patients. They investigated the expression of miRNA-99a, miRNA-100, and miRNA-199b, which target the mTOR kinase. A combined signature of miRNA-99a and miRNA-199b in plasma samples resulted in 88% sensitivity and 93% specificity, indicating a good diagnostic potential [12]. Despite these findings, they were not applied in the clinical setting [13]. In this respect, EVs arise as a source of biomarkers with an unexploited potential. They can be isolated from bodily fluids, such as saliva, blood, urine, malignant pleural effusion, and ascitis [14]. In EC, miRNAs isolated from EVs have been scarcely studied. Akhil et al. evaluated the potential of the miRNA content of urine-derived EVs as a diagnostic biomarker in EC patients [15], and Hanzi Xu et al. isolated EVs from serum samples and identified 209 upregulated and 66 downregulated circular RNA (circRNAs) in EVs from serum of patients with EC compared with those from serum of healthy controls [16].

Although plasma, serum, and urine biopsies are the most common liquid biopsies, the use of proximal bodily fluids as a source of biomarkers has attracted the attention of the biomarker research community. Proximal bodily fluids, such as uterine fluid for EC, offer an improved representation of the molecular alterations that take place in the tumor [17]. The peritoneal fluid is another proximal fluid of EC; however, this type of proximal fluid has not been yet exploited to investigate EC-related biomarkers or any other cancer originating within the peritoneal cavity.

To the best of our knowledge, our study is the first to report the value of this proximal fluid for the identification of miRNAs associated with EVs in EC. Importantly, this study identified the dysregulation of 114 miRNAs, among which miRNA-383-5p, miRNA-10b-5p, miRNA-34c-3p, miRNA-449b-5p, miRNA-34c-5p, miRNA-200b-3p, miRNA-2110, and miRNA-34b-3p are of special interest, as they demonstrated a high classification potential. Interestingly, some of these miRNAs were found in previous EC studies. In concordance with our study, miRNA-10b and miRNA-34b were found to be downregulated in endometrial serous adenocarcinoma versus normal endometrial tissue, indicating that these miRNAs might also be associated with the aggressive subtype of the serous EC. In fact, in that study, reduced miRNA-10b expression was found to be significantly correlated with shorter overall survival [18]. MiRNA-34 has been described as a fundamental regulator of tumor suppression; it controls multiple protein targets involved in the cell cycle and apoptosis, and was associated with metastasis and chemoresistance [19]. In contrast to our study, miRNA-200b was found to be upregulated in endometrial serous adenocarcinoma versus normal endometrial tissue [18]. However, it has been reported to be downregulated in various human malignancies, and its function has been postulated to be oncogenic (i.e., involved in proliferation, motility, apoptosis, stemness, and the epithelial-to-mesenchymal transition) [20]. Interestingly, accumulating evidence in the field of endometriosis suggests that apoptosis that occurs in the peritoneal cavity may play a pivotal role in addressing the immune homeostasis in the peritoneal microenvironment [21]. This causes scavenging mechanisms to fail, allowing for the survival of endometriotic cells in patients with endometriosis [22]. In the context of endometrial cancer, we speculate that EVs derived from endometrial cancer cells might reach the peritoneal cavity and target the mesothelial liner cells of the peritoneum and ovaries to modulate the immune homeostasis and create a more favorable milieu to metastasize. In fact, most of the metastasis associated with endometrial cancer occurs either in the vagina, in the lymph nodes, or within the peritoneal cavity. Several of the miRNAs identified in our study are related to tumor progression in endometrial cancer or in other tumor types. It is possible that endometrial cancer cells

may spread into the peritoneum and the ovaries through the Fallopian tube, and develop peritoneal metastasis and ovarian metastasis in the absence of lympho-vascular space invasion. It is important to note that normal endometrium may spread into the peritoneum and the ovaries through retrograde menstruation, and give rise to endometriosis. Metastasis that originates through this pathway may be associated with indolent behavior. A subset of ECs from ovarian metastasis are associated with such a good prognosis that they were interpreted as synchronous tumors [23]. Next-generation sequence analysis has confirmed that they are metastatic. Assessing transtubal exosomal release from endometrial cancer may help us to understand the mechanisms involved in this type of indolent metastasis.

The study leaves some questions open. This study enabled us to identify a large number of dysregulated miRNAs associated with EVs in the peritoneal lavage of EC patients. We think that the EVs that we have identified come from endometrial and mesothelial cells in the EC group, and mostly from mesothelial cells in the control group. The comparative analysis of EC patients with non-cancer patients with ascites suggests that these selected miRNAs come from EVs of EC tissue. However, there is obviously the probability that a subset of EVs came from an inflammatory reaction associated with EC and some other factors distinct from the EC pathology, such as the source of EVs (peritoneal lavage vs. ascitic fluids), the surgery (after induction of general anesthesia), and the control samples, which were obtained by means of paracentesis (with local anesthesia). Nevertheless, these promising biomarkers should be further validated as well as combined in order to increase the already excellent accuracy of each individual miRNA. This should be done in an independent study involving a larger cohort of EC patients versus a control group with a higher biologic variability, including, for example, patients with leiomyomas or women requiring tubal ligation for definitive contraception. Although we tested whether or not differentially expressed miRNAs were dependent on gender, further studies should include only female controls. Moreover, further research should be directed to an evaluation of the prognostic potential of each specific dysregulated miRNA, as this might help to guide the surgical treatment of EC patients.

4. Materials and Methods

4.1. Patients and Ascitic Fluid and Peritoneal Lavage Collection

All subjects provided informed consent before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by The Clinical Research Ethics Committee of Hospital Arnau de Vilanova in Lleida, Spain (Approval number: CEIC-1630). Samples were obtained with support from the IRBLleida Biobank (B.0000682) and Plataforma biobancos PT17/0015/0027. Ascitic fluids and peritoneal lavages were extracted from a cohort of 50 patients, corresponding to 25 control patients with decompensated cirrhosis and 25 patients with EC who underwent curative surgery. In the control patients, the collection of ascitic fluids was performed as follows: Ascitic fluids were aspirated using 18 or 21G needles (for diagnostic paracentesis) or an over-the-needle catheter device (for therapeutic paracentesis). The procedure was performed under sterile conditions, the needle insertion site was selected by ultrasound guidance, and the skin and parietal peritoneum were previously anesthetized with 2% mepivacine. A total of 100 mL of ascitic fluid was gently aspirated, collected into a 50 mL tube, and stored at −80 °C until use. In EC patients, the collection of peritoneal lavage was performed during surgery, once the abdominal cavity was opened and prior to any manipulation of the uterus. A total of 100 mL of physiological saline was instilled into the abdominal cavity with a 50 mL syringe, mobilizing patients for the correct distribution of saline, which was then extracted with a 50 mL syringe connected to a 14-gauge aspiration needle. The peritoneal lavage was gently aspirated. A volume ranging from 50 to 100 mL was collected and stored at −80 °C until use. The clinical features of each patient are listed in Supplementary Table S1.

4.2. EV Isolation

EVs were isolated with a differential centrifugation method as previously described [24] with slight modifications. Briefly, ascitic fluids and peritoneal lavages were centrifuged at 300× *g* for 10 min, followed by centrifugation at 2500× *g* for 20 min at the moment that the sample was collected, and frozen at −80 °C. Then, samples were centrifuged at 10,000 *g* for 30 min (Thermo Scientific Heraeus MultifugeX3R Centrifuge (FiberLite rotor F15-8x-50c)). The supernatant was filtered through 0.22 µm filters (Merck Millipore), and the obtained sample was transferred to ultracentrifuge tubes (Beckman Coulter), which were filled with phosphate-buffered saline (PBS), to perform two consecutive ultracentrifugation steps at 100,000 *g* for 2 hours each on a Thermo Scientific Sorvall WX UltraSeries Centrifuge with an AH-629 rotor. The pellet containing the EVs was resuspended in 50 µL of PBS. From those, 5 µL were isolated for nanoparticle tracking analysis (NTA) and quantification, and the rest was frozen at −80 °C with 500 µL of Qiazol for RNA extraction.

4.3. Nanoparticle Tracking Analysis

The size and number of EVs were determined using a Nanosight LM10 instrument equipped with a 405 nm laser and a Hamamatsu C11440 ORCA-Flash 2.8 camera (Hamamatsu) with Nanoparticle Tracking Analysis (NTA, Malvern Instruments, UK). Each sample was diluted appropriately with Milli-Q water (Milli-Q Synthesis, Merck Millipore, MA, USA) to give counts in the linear range of the instrument. The particles in the laser beam underwent Brownian motion, and a video was recorded for 60 s in triplicate. The analysis was performed by following the manufacturer's instructions, and data were analyzed using version 2.3 of the NTA software.

4.4. Total RNA Extraction and OpenArray Analysis

Total RNA, including miRNAs and other RNAs, was isolated from the EV samples using the miRNeasy MiniKit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA from EVs was eluted with 30 µL of Nuclease-free water (Qiagen, Hilden, Germany). MiRNA expression was determined using a TaqMan OpenArray Human MicroRNA Panel, QuantStudio 12K Flex (Catalog number: 4470187, Thermo Fisher Scientific, Waltham, MA, USA), a fixed-content panel containing 754 well-characterized human miRNA sequences from the Sanger miRBase v14, and according to the manufacturer's instructions. Reverse transcription (RT) was performed on 2 µL RNA using Megaplex™ Primer Pools A and B and the supporting TaqMan® MicroRNA Reverse Transcription Kit as follows: 15 min at 42 °C and 5 min at 85 °C. Then, 5 µL of the resulting cDNA was preamplified prior to real-time PCR analysis using Megaplex™ PreAmp Pools and the TaqMan® PreAmp Master Mix using the following conditions: one single step at 95 °C for 5 min, 20 cycles of a two-step program (3 sec, 95 °C and 30 sec, 60 °C) followed by a single cycle of 10 min at 99 °C to inactivate the enzyme. The preamplified products were diluted to 1:20 in 0.1× TE buffer pH 8.0, and mixed in a 1:1 ratio with TaqMan® OpenArray® Real-Time PCR Master Mix in the 384-well OpenArray® Sample Loading Plate. TaqMan® OpenArray® MicroRNA Panels were automatically loaded using the AccuFill™ System.

4.5. Preprocessing and Differential Expression Analysis

All bioinformatics analyses were performed with BioConductor (version 3.7) [25] in the R statistical environment (version 3.5.0) [26]. For the data preprocessing, the HTqPCR (version 1.34) R package [27] was used. Probes that had a "Cycle threshold" (Ct) value of 40 in all samples were removed. Samples in which more than 80% of the probes had a Ct value greater than 40 were retained. To assure comparability across samples, the Ct values were delta-normalized. The average Ct values of the probes hsa−miR−150−5p, hsa−let−7g-5p, hsa−miR−598−3p, and hsa−miR−361−3p were used for normalization. These probes had Ct values of 40 in a maximum of three samples and the lowest interquartile range across all samples. The differential expression analysis was carried out with the

empirical Bayes approach on linear models using the limma (version 3.36) R Package [28]. Results were corrected for multiple testing using the False Discovery Rate (FDR) [29].

4.6. Development of Predictors

For predictive analysis, the whole patient cohort was randomly divided into training and validation sets with a ratio of 3:2. Calculated (with the limma R Package) relative miRNA expression values were used as input variables into a logistic regression model between groups. Each miRNA (adjusted p-value <0.05) was fitted in the logistic regression model to differentiate the EC and the control patient groups in the training set, and its classification ability was evaluated using the area under the ROC curve (AUC), accuracy, sensitivity, and specificity values on the validation set. The procedures for the division of the patient cohort into training and validation sets and fitting the logistic model were repeated 500 times and statistics were collected.

4.7. miRNA Target Gene Prediction and Bioinformatics Analysis

miRNA target genes were predicted using the Predictive Target Module of the miRWalk2.0 online software [30] (https://goo.gl/ajG9ja). Only genes for which miRNAs recognize a minimum 7 bp seed length, seed start at position 1, and sequence localize at 3´UTR were considered as valid targets. Moreover, to improve target gene prediction accuracy, we considered only those transcripts that were predicted in at least eight out of the 12 databases (miRWalk, miRanda, MicroT4, miRDB, miRMap, miRBridge, miRNAMap, PICTAR2, RNA22, PITA, TargetScan, and RNAhybrid) presented in the miRWalk2.0 tool.

The online Panther software [31] (http://www.pantherdb.org/) was used for the Gene Ontology (GO) functional analysis to analyze the potential functions of the predicted target genes. Biological process (BP) and molecular function (MF) GO terms were analyzed and plotted.

5. Conclusions

Thanks to this study, we have demonstrated that the use of EV-associated miRNAs of ascitic fluid from control patients and peritoneal lavages from EC patients are an untapped source of biomarkers. Specifically, we identified 114 dysregulated miRNAs, and, among those, miRNA-383-5p, miRNA-10b-5p, miRNA-34c-3p, miRNA-449b-5p, miRNA-34c-5p, miRNA-200b-3p, miRNA-2110, and miRNA-34b-3p were highlighted as promising biomarkers of EC with an AUC value higher than 0.90.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6694/11/6/839/s1, Figure S1: EV characterization. (A) Box-plot representing the average mode of EVs isolated from the peritoneal lavage and the ascitic fluid of EC and control patients, respectively (Mean ± SD), measured by Nanoparticle Tracking Analysis. (B) Size distribution and concentration of isolated EVs of a peritoneal lavage of an EC patient (left) and an ascitic fluid of a control patient (right), measured by Nanoparticle Tracking Analysis. Table S1: Clinicopathological characteristics of all patients. Clinical characteristics of the total cohort of patients recruited in the study.

Author Contributions: Conceptualization, E.C. and X.M.-G.; methodology, B.R.-C., C.P.M., S.B., S.G., M.R.-M., E.G., X.G.-T., I.L., I.H., and J.M.P.; software, J.P. and S.B.; validation, J.P., S.B., E.C., and B.R.-C.; formal analysis, S.B., J.P., C.P.M., and B.R.-C.; investigation, B.R.-C.; resources, J.M.F.-P., X.M.-G., and E.C.; data curation, S.B. and J.P.; writing—original draft preparation, B.R.-C., C.P.M., and J.P.; writing—review and editing, C.P.M., J.P., E.C., and X.M.-G.; visualization, X.M.-G. and E.C.; supervision, X.M.-G. and E.C.; project administration, X.M.-G. and E.C.; funding acquisition, A.G.-M., X.M.-G., and E.C.

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Conflicts of Interest: The authors declare no conflicts of interest.

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Cancers **2019**, *11*

Supplementary Materials: EV-Associated miRNAs from Peritoneal Lavage is a Source of Biomarkers in Endometrial Cancer

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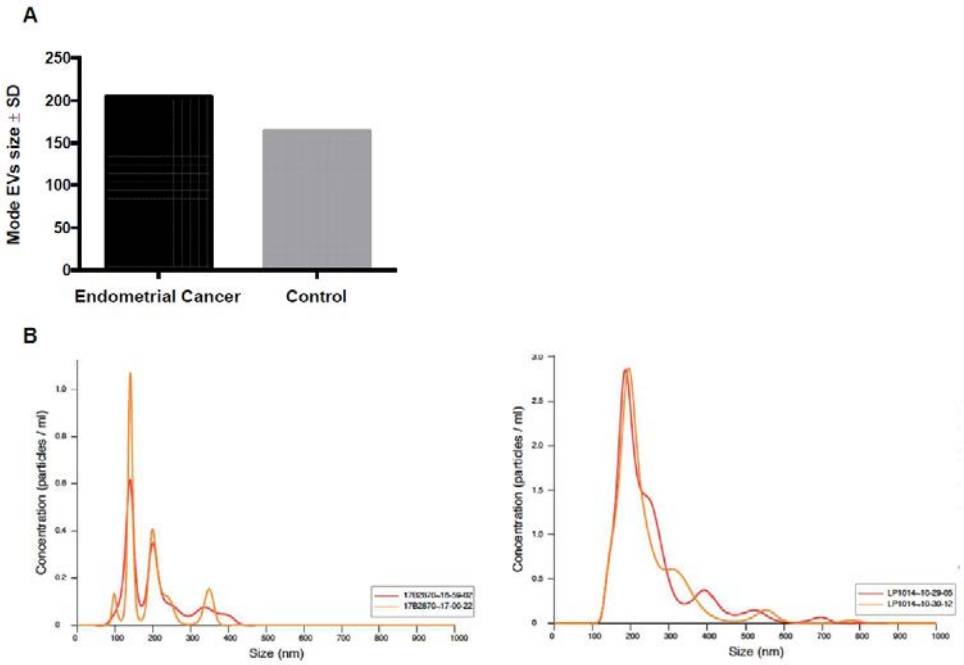

Figure S1. EVs characterization. (**A**) Box-plot representing the average mode of EVs isolated from the peritoneal lavage and ascitic fluid of EC and control patients, respectively (Mean ± SD); measured by Nanoparticle Tracking Analysis. (**B**) Size distribution and concentration of isolated EVs of a peritoneal lavage of a EC patient (left) and a ascitic fluid of a control patient (right), measured by Nanoparticle Tracking Analysis.

Patient	Pathology	Age	Gender	Histological subtype	Histological grade	Metastasis	Primary tumor localization	Medical treatment
1	Hepatic cirrhosis	74	Male					
2	Hepatic cirrhosis	65	Male					
3	Hepatic cirrhosis	67	Male					
4	Hepatic cirrhosis	60	Male					
5	Hepatic cirrhosis	56	Male					
6	Hepatic cirrhosis	50	Male					
7	Hepatic cirrhosis	54	Male					
8	Heart failure	58	Male					
9	Hepatic cirrhosis	65	Male					
10	Hepatic cirrhosis	63	Male					
11	Hepatic cirrhosis	65	Male					
12	Hepatic cirrhosis	74	Female					
13	Hepatic cirrhosis	62	Male					
14	Hepatic hydrothorax	68	Male					
15	Hepatic cirrhosis	52	Female					
16	Hepatic cirrhosis	78	Female					
17	Hepatic cirrhosis	60	Male					
18	Hepatic cirrhosis	60	Male					
19	Hepatic cirrhosis	51	Male					
20	Hepatic cirrhosis	72	Female					
21	Hepatic cirrhosis	54	Female					
22	Hepatic cirrhosis	90	Male					
23	Hepatic cirrhosis	73	Male					
24	Hepatic cirrhosis	73	Male					
25	Hepatic cirrhosis	70	Male					
26	Endometrial Cancer	57	Female	EEC	III	Peritoneum	Uterus	Chemotherapy + Radiotherapy
27	Endometrial Cancer	65	Female	EEC	I	No	Uterus	No
28	Endometrial Cancer	46	Female	EEC	I	No	Uterus	No
29	Endometrial Cancer	54	Female	EEC	I	No	Uterus	No
30	Endometrial Cancer	60	Female	EEC	I	No	Uterus	Brachytherapy
31	Endometrial Cancer	74	Female	NEEC	III	No	Uterus	No
32	Endometrial Cancer	52	Female	EEC	I	No	Uterus	No
33	Endometrial Cancer	53	Female	EEC	I	No	Uterus	No
34	Endometrial Cancer	61	Female	NEEC		No	Uterus	No
35	Endometrial Cancer	61	Female	EEC	I	No	Uterus	No
36	Endometrial Cancer	70	Female	EEC	I	No	Uterus	No
37	Endometrial Cancer	78	Female	EEC	II	No	Uterus	No
38	Endometrial Cancer	49	Female	EEC		No	Uterus	No
39	Endometrial Cancer	60	Female	EEC	I	No	Uterus	No
40	Endometrial Cancer	77	Female	NEEC	III	Peritoneum and Ovaries	Uterus	No
41	Endometrial Cancer	61	Female	EEC	II	No	Uterus	No
42	Endometrial Cancer	80	Female	NEEC	III	Pelvis and Lung	Uterus	Palliative care
43	Endometrial Cancer	70	Female	EEC	II	No	Uterus	Brachytherapy
44	Endometrial Cancer	61	Female	EEC	I	No	Uterus	No
45	Endometrial Cancer	58	Female	EEC	II	No	Uterus	Brachytherapy
46	Endometrial Cancer	55	Female	EEC	III	No	Uterus	Brachytherapy
47	Endometrial Cancer	62	Female	EEC	I	No	Uterus	No
48	Endometrial Cancer	73	Female	EEC	I	No	Uterus	No
49	Endometrial Cancer	55	Female	EEC	I	No	Uterus	No
50	Endometrial Cancer	77	Female	NEEC	III	No	Uterus	Out of follow-up

* EEC: endometrioid endometrial carcinoma
* NEEC: non-endometrioid endometrial carcinoma
* I, II and III: Grade I, II and III

CHAPTER II

EV-Associated miRNAs from Peritoneal Lavage as a Potential Diagnostic Biomarkers in Colorectal Cancer

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RESEARCH

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EV-associated miRNAs from peritoneal lavage as potential diagnostic biomarkers in colorectal cancer

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Abstract

Background: Colorectal cancer (CRC) is the third leading cause of cancer-related mortality worldwide. Current systematic methods for diagnosing have inherent limitations so development of a minimally-invasive diagnosis, based on the identification of sensitive biomarkers in liquid biopsies could therefore facilitate screening among population at risk.

Methods: In this study, we aim to develop a novel approach to identify highly sensitive and specific biomarkers by investigating the use of extracellular vesicles (EVs) isolated from the peritoneal lavage as a source of potential miRNA diagnostic biomarkers. We isolated EVs by ultracentrifugation from 25 ascitic fluids and 25 peritoneal lavages from non-cancer and CRC patients, respectively. Analysis of the expression of EV-associated miRNAs was performed using Taqman OpenArray technology through which we could detect 371 miRNAs.

Results: 210 miRNAs were significantly dysregulated (adjusted p value < 0.05 and abs(logFC) ≥ 1). The top-10 miRNAs, which had the AUC value higher than 0.95, were miRNA-199b-5p, miRNA-150-5p, miRNA-29c-5p, miRNA-218-5p, miRNA-99a-3p, miRNA-383-5p, miRNA-199a-3p, miRNA-193a-5p, miRNA-10b-5p and miRNA-181c-5p.

Conclusions: This finding opens the avenue to the use of EV-associated miRNA of peritoneal lavages as an untapped source of biomarkers for CRC.

Keywords: Colorectal cancer, Biomarkers, Diagnostic, miRNAs, Ascitic fluid, Peritoneal lavage, Liquid biopsy, Extracellular vesicles, Colon cancer

Background

Colorectal cancer (CRC) is the third most common type of malignant tumor and the third leading cause of cancer-related mortality worldwide among men and women [1]. The overall survival of colorectal cancer is 65%, but this is highly dependent upon the disease stage at diagnosis, and ranges from a 90% of 5-year survival rate for cancers detected at the localized stage (40% of the cases) and 70% for regional (35% of the cases) to 15% for distant metastatic cancer (20% of the cases) [2]. Current systematic methods for diagnosis, such as fecal occult blood test and flexible sigmoidoscopy, help to reduce mortality by removing precursor lesions and making diagnosis at an earlier stage. However, these techniques have inherent limitations, such as low sensitivity and invasiveness for patients, respectively; and

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the burden of disease and mortality is still high [3]. Serum tumor markers CA19-9 and CEA have been used for detection of many types of cancer, but their sensitivity for the detection of CRC is low [4]. Therefore, development of a minimally-invasive diagnosis, based on the identification of sensitive biomarkers in liquid biopsies could therefore facilitate screening among population at risk of CRC, impact on early detection, and thus, decrease CRC-related mortality.

MiRNAs are a highly conserved family of endogenous non-coding and single-stranded RNAs that are 19–24 nucleotides in length [5]. Generally, miRNAs negatively regulate gene expression via binding to the 3′-untranslated region (3′-UTR) of their target double-stranded mRNA that results in transcriptional repression or mRNA degradation upon dicer complex [6]. miRNAs have been implicated in development and progression of CRC by functioning as oncogenes and tumor suppressors [7]. Recent studies demonstrated that miRNAs are secreted from various cells, including cancer cells, into bodily fluids such as blood, urine, breast milk, and saliva, either as free miRNAs or via extracellular vesicles (EVs) [4].

EVs are 20–200 nm membrane vesicles released by either directly from plasma membranes, or from intracellular multivesicular bodies by their fusion with the cell membrane. Their function is to mediate intercellular communication, influencing the recipient cell function. Importantly, EVs have awakened the interest of the scientific community as a source of biomarkers, mainly because they carry a broad range of bioactive material (proteins, metabolites, RNA, miRNA, etc.) and this material is well-protected owing to the EVs lipid bilayer membrane, even if EVs are extracted from circulating or proximal body fluids [8].

Herein, we investigated the use of EVs isolated from the peritoneal lavage, a proximal fluid in CRC patients, as a source of potential diagnostic biomarkers. To do so, we conducted miRNA-profiling of EVs isolated from peritoneal lavages of surgical CRC patients and ascitic fluids of non-cancer patients by using the TaqMan OpenArray Human MicroRNA Panel. We unveiled the most relevant individual miRNAs for diagnosing CRC and characterized the biological and molecular landscape of the CRC milieu. The study was conceived as a proof of concept investigation to demonstrate the feasibility of peritoneal lavage as a source of EV-associated miRNAs in patients with CRC.

Methods

Patients and ascitic fluid and peritoneal lavages collection

Participants in the study attended to the Hospital Arnau de Vilanova in Lleida, Spain. The Clinical Research Ethics Committee of the hospital approved the study and all the participating patients provided a signed informed consent. Ascitic fluids and peritoneal lavages were extracted from a cohort of 50 patients, corresponding to 25 control patients with decompensated cirrhosis, and 25 patients with CRC who underwent curative surgery. In control patients, the collection of ascitic fluid was aspirated using 18 or 21G needles (for diagnostic paracentesis) or an over-the-needle catheter device (for therapeutic paracentesis). The procedure was performed under sterile conditions, the site of needle insertion was selected by ultrasound guidance, and skin and parietal peritoneum were previously anesthetized with 2% mepivacine. A total of 100 mL of ascitic fluid was gently aspirated, collected into a 50 mL tube and stored at −80 °C. In CRC patients, the collection of peritoneal lavage was performed before the surgery, once the abdominal cavity has been opened and prior to any manipulation of the colon. A total of 100 mL of physiological saline were instilled into the abdominal cavity with a 50 mL syringe, mobilizing patients for the correct distribution of saline, which was then extracted with a 50 mL syringe connected to a 14-gauge aspiration needle. The peritoneal lavage was gently aspirated. A volume ranging from 50 to 100 mL was collected and stored at −80 °C. The clinical features of each patient are listed in Additional file 1: Table S1.

EVs isolation

EVs were isolated with a differential centrifugation method as previously described [9] with slight modifications. Briefly, ascitic fluids and peritoneal lavages were centrifuged at 300×*g* for 10 min, followed by a centrifugation at 2500×*g* for 20 min and a centrifugation of 10,000*g* for 30 min (Thermo Scientific Heraeus MultifugeX3R Centrifuge (FiberLite rotor F15-8x-50c)). The supernatant was then filtered through 0.22 µm filters (Merck Millipore) and the sample obtained was transferred to ultracentrifuge tubes (Beckman Coulter) and filled with PBS to perform two consecutive ultracentrifugation steps at 100,000*g* for 2 h each on a Thermo Scientific Sorvall WX UltraSeries Centrifuge with an AH-629 rotor. The pellet containing the EVs was resuspended in 50 µL of PBS. From those, 5 µL were isolated for nanoparticle tracking analysis (NTA) and quantification, and the rest was frozen at −80 °C with 500 µL of Qiazol for RNA extraction.

Nanoparticle tracking analysis

Size and number of EVs was determined using a Nanosight LM10 instrument equipped with a 405 nm laser and a Hamamatsu C11440 ORCA-Flash 2.8 camera

(Hamamatsu) with Nanoparticle Tracking Analysis (NTA, Malvern Instruments, UK). Each sample was diluted appropriately with Milli-Q water (Milli-Q Synthesis, Merck Millipore, Massachusetts, USA) to give counts in the linear range of the instrument. The particles in the laser beam undergo Brownian motion, and a video was recorded for 60 s in triplicate. Analysis was performed following manufacturer's instructions and data were analyzed using the version 2.3 of the NTA-software.

Total RNA extraction and OpenArray analysis

Total RNA, including miRNAs and other RNAs, was isolated from the EVs samples using the miRNeasy Mini-Kit (Qiagen) according to manufacturer's protocol. RNA from EVs was eluted with 30 µL of Nuclease-free water (Ambion). MiRNA expression was performed using TaqMan OpenArray Human MicroRNA Panel, QuantStudio 12 K Flex (Catalog number: 4470187, Thermo Fisher Scientific), a fixed-content panel containing 754 well-characterized human miRNA sequences from the Sanger miRBase v14 and according to the manufacturer's instructions. Reverse transcription (RT) was performed on 2 µL RNA using Megaplex™ Primer Pools A and B and the supporting TaqMan® MicroRNA Reverse Transcription Kit as follows: 15 min at 42 °C and 5 min at 85 °C. Then, 5 µL of the resulting cDNA was preamplified prior to real-time PCR analysis using Megaplex™ PreAmp Pools and the TaqMan® PreAmp Master Mix using the following conditions: one single step at 95 °C during 5 min, 20 cycles of a two-steps program (3 s, 95 °C and 30 s, 60 °C) followed by a single cycle of 10 min at 99 °C to inactivate the enzyme. The preamplified products were diluted 1:20 in 0.1× TE buffer pH8.0, and mixed in 1:1 with TaqMan® OpenArray® Real-Time PCR Master Mix in the 384-well OpenArray® Sample Loading Plate. TaqMan® OpenArray® MicroRNA Panels were automatically loaded using the AccuFill™ System.

Preprocessing and differential expression analysis

All bioinformatics analysis was performed with the BioConductor (version 3.7) [10] project in the R statistical environment (version 3.5.0) [11]. For the data preprocessing, the HTqPCR (version 1.34) R package [12] was used. Probes that had a "Cycle threshold" (Ct) value of 40 in all samples were removed. Further samples in which more than 80% of the probes had a Ct value above 40 were retained. To assure comparability across samples, the Ct values were delta normalized. The average Ct values of the probes $hsa-miR-150-5p$, $hsa-let-7g\text{-}5p$, $hsa-miR-598-3p$, and $hsa-miR-361-3p$ were used for normalization. These probes had the Ct values of 40 in a maximum of three samples, and the lowest interquartile range across samples. Differential expression analysis was carried out with an empirical Bayes approach on linear models, using the limma (version 3.36) R Package [13]. Results were corrected for multiple testing using the False Discovery Rate (FDR) [14].

Development of predictors

For predictive analysis, the whole patient cohort was randomly divided into training and validation sets with the 3:2 ratio. Calculated (with the limma R Package) relative miRNA expression values were used as input variables to a logistic regression model between groups. Each miRNA (adjusted p-value < 0.05) was fitted in the logistic regression model to differentiate the CRC and the control patients groups in the training set and its classification ability was evaluated using the AUC (area under the ROC curve), accuracy, sensitivity, and specificity values on the validation set. The procedure from division into training and validation sets and fitting the logistic model was repeated 500 times and statistics were collected.

miRNA target genes prediction and bioinformatics analysis

miRNAs target genes were obtained using the Predictive Target Module of miRWalk2.0 online software [15] (https://goo.gl/ajG9ja), considering the following parameters: 3´UTR localization, miRNA seed start at position 1 and minimum 7 bp seed length. To improve the accuracy of target gene prediction, only those transcripts that were predicted in at least 8 out of the 12 databases were considered (miRWalk, miRanda, MicroT4, miRDB, miRMap, miRBridge, miRNAMap, PICTAR2, RNA22, PITA, TargetScan, and RNAhybrid). Gene Ontology (GO) functional analysis were used to analyze the potential functions of the predicted target genes, using the online Panther software [16] (http://www.pantherdb.org/). Biological process (BP) and molecular function (MF) GO terms were analyzed and plotted.

Results

We analyzed the miRNA profile of EVs isolated from the ascitic fluid of 25 control individuals and peritoneal lavage of 25 CRC patients. Additional file 2: Figure S1 illustrates the workflow that was followed in this study. The quality of EVs isolated from the ascitic fluids and peritoneal lavages was measured by size distribution and concentration by Nanoparticle Tracking Analysis, demonstrating that we analyzed a population mostly enriched in small EVs but also containing a low representation of larger vesicles (Additional file 3: Figure S2). MiRNAs were extracted from EVs for a systematic miRNA expression analysis using the Taqman OpenArray technology, through which we detected 371 out of the 754 miRNAs (49.2%) present in the OpenArray. Probes that had the

Ct value of 40 in all samples and samples in which more than 80% of the probes had the Ct value above 40 were removed, resulting in 355 miRNAs from 22 control and 19 CRC patients analyzed for the differential expression analysis (Table 1).

The differential expression analysis between cancer and control cases yielded a list of 210 miRNAs that were significantly dysregulated (adj. p-value < 0.05 and logFC lower or higher than 1). Among those, 207 miRNA were found to be downregulated and 3 miRNA were upregulated in CRC patients. To evaluate whether these miRNAs can be used as diagnostics biomarkers, we performed a predictive analysis using the logistic modeling. Ten miRNAs demonstrated predictive performance at the AUC values higher than 0.95: miRNA-199b-5p, miRNA-150-5p, miRNA-29c-5p, miRNA-218-5p, miRNA-99a-3p, miRNA-383-5p, miRNA-199a-3p, miRNA-193a-5p, miRNA-10b-5p and miRNA-181c-5p (Table 2; Fig. 1). All those miRNAs were downregulated from 3.52 to 12.82 in the $\log_2$ scale with adjusted p-value lower than 1.56E−05, except miRNA-150-5p which was upregulated (adjusted p-value 3.41E−04). In Table 3, studies reporting an association between each of these top-10 miRNAs and CRC are described based on a search of Pubmed for each miRNA and the word "colorectal cancer". Although there are some controversies among the different studies, most of the miRNA dysregulations observed in our study are concordant with the observations reported by other authors, either in tissue, plasma or stool samples. MiRNA-199b-5p, miRNA-29c-5p, and miRNA-99a-3p have never been reported previously in association to CRC.

Table 1 Clinicopathological characteristics of patients

	Colon Cancer	Control
Age		
Median	74	65
Minimum	50	52
Maximum	88	90
Gender		
Female	12	4
Male	7	18
Pathology		
Colon cancer	19	–
ADC low grade	15	–
ADC other types	4	–
Hepatic cirrhosis	–	20
Others	–	2

Clinical characteristics of the final cohort of patients included in the study after data normalization

ADC adenocarcinoma

To further understand the milieu generated by CRC EVs, we performed a bioinformatics study to first unveil the predicted transcripts that are regulated by all the differential miRNAs, and then assess the biological processes and molecular functions in which they participate. A total of 9358 transcripts were found to be regulated by the 210 miRNA differentially expressed. Figure 2 shows the number and most frequently regulated transcripts predicted for each dysregulated miRNA. To comprehensively integrate the properties of all target transcripts, they were studied using Gene Ontology (GO). The most enriched biological processes in CRC EVs were metabolic processes (24.3%), mostly including biosynthetic process, organic substance metabolomic process and cellular metabolic process; biological regulation (22.5%); cellular processes (10.7%), signal transduction, cellular component organization and cellular metabolic process; and cellular component organization or biogenesis, including cellular component organization (Fig. 3a). In relation to the most altered molecular functions in CRC EVs, the Gene Ontology (GO) analysis revealed that many targeted transcripts were found to be involved in binding (37.8%), including protein binding and organic cyclic compound binding; and in catalytic activity (31.2%), including catalytic activity, and protein and hydrolase activity (Fig. 3b).

Discussion

In this study we investigated, for the first time, the miRNA content of EVs isolated from peritoneal lavages and ascitic liquid of CRC and control patients, respectively. Our study shows that EV-associated miRNAs can be consistently extracted from peritoneal lavages and ascitic liquids and that miRNA expression profiles can indicate and represent the status of CRC patients. The EV-associated miRNA were analyzed by Taqman OpenArray technology and the differential expression analysis yielded a list of 210 miRNAs that were significantly dysregulated in CRC patients, being downregulated the 98.57% of the altered miRNAs.

The finding that miRNAs are dysregulated in CRC patients is known, as many studies have reported this previously, mostly in tissue specimens [17] but also in different body fluids. In CRC, most of the studies use plasma or serum as it is the most common, easy-to-handle, accessible liquid biopsy. The first report detected 69 miRNAs in serum of CRC patients but not in serum of normal controls [18]. Since then, several studies have identified miRNA upregulation or downregulation in plasma or serum samples [17] including studies that have focus on the search of biomarkers in miRNAs dysregulated in the vesicular fraction of the serum or plasma of CRC patients. Hiroko Ogata-Kawata et al. [4] analyzed the EV-associated miRNA

Table 2 miRNA transcripts displaying a significant differential expression in patients with CRC compared to control patients

miRNA	LogFC	p-value	Adj. p-value	AUC	AUC 95% CI_lower	AUC 95% CI_upper	Accuracy	Sensitivity	Specificity
hsa-miR-199b-5p_478486_mir	− 12.82	2.59E−08	9.85E−07	1	1	1	0.967	0.968	0.964
hsa-miR-150-5p_477918_mir	2.58	7.10E−05	3.41E−04	0.978	0.959	0.996	0.919	0.936	0.899
hsa-miR-29c-5p_478005_mir	− 2.93	2.78E−08	9.85E−07	0.973	0.954	0.991	0.943	0.943	0.944
hsa-miR-218-5p_477977_mir	− 8.16	6.51E−07	8.25E−06	0.97	0.945	0.995	0.913	0.905	0.921
hsa-miR-99a-3p_479224_mir	− 4.89	1.51E−08	8.95E−07	0.97	0.95	0.99	0.94	0.976	0.9
hsa-miR-383-5p_478079_mir	− 8.33	3.55E−15	1.26E−12	0.968	0.952	0.985	0.939	0.94	0.938
hsa-miR-199a-3p_477961_mir	− 6.16	2.84E−09	2.65E−07	0.968	0.942	0.994	0.905	0.92	0.887
hsa-miR-193a-5p_477954_mir	− 3.62	1.32E−06	1.56E−05	0.962	0.932	0.991	0.873	0.852	0.897
hsa-miR-10b-5p_478494_mir	− 2.79	2.58E−07	4.17E−06	0.957	0.93	0.983	0.871	0.875	0.866
hsa-miR-181c-5p_477934_mir	− 3.52	1.23E−05	8.74E−05	0.952	0.929	0.974	0.833	0.859	0.803
hsa-miR-708-5p_478197_mir	− 6.45	9.72E−08	2.29E−06	0.946	0.917	0.975	0.877	0.834	0.926
hsa-miR-125b-5p_477885_mir	− 2.35	9.27E−07	1.13E−05	0.946	0.918	0.974	0.885	0.884	0.885
hsa-miR-140-5p_477909_mir	− 5.82	4.59E−05	2.39E−04	0.943	0.919	0.968	0.817	0.825	0.807
hsa-miR-451a_478107_mir	− 8.35	1.34E−07	2.64E−06	0.942	0.913	0.972	0.843	0.881	0.8
hsa-miR-148b-3p_477824_mir	− 3.05	1.42E−07	2.66E−06	0.942	0.916	0.968	0.834	0.853	0.813
hsa-miR-130a-3p_477851_mir	− 2.62	1.85E−06	2.00E−05	0.94	0.909	0.972	0.861	0.884	0.835
hsa-miR-214-3p_477974_mir	− 7.59	1.19E−07	2.49E−06	0.937	0.901	0.972	0.896	0.94	0.846
hsa-miR-10a-5p_479241_mir	− 2.19	1.44E−02	2.82E−02	0.937	0.906	0.969	0.897	0.904	0.889
hsa-miR-497-5p_478138_mir	− 3.68	2.13E−04	8.08E−04	0.936	0.911	0.961	0.814	0.829	0.797
hsa-miR-143-3p_477912_mir	− 3.15	1.58E−06	1.81E−05	0.936	0.906	0.965	0.86	0.868	0.85
hsa-miR-20a-5p_478586_mir	− 2.79	5.14E−08	1.30E−06	0.933	0.901	0.964	0.877	0.906	0.843
hsa-miR-29c-3p_479229_mir	− 3.55	2.14E−04	8.08E−04	0.931	0.897	0.965	0.86	0.84	0.883
hsa-miR-17-5p_478447_mir	− 3.68	4.83E−05	2.41E−04	0.93	0.893	0.966	0.874	0.898	0.847
hsa-miR-486-5p_478128_mir	− 11.10	2.21E−07	3.73E−06	0.929	0.899	0.958	0.853	0.818	0.893
hsa-miR-145-5p_477916_mir	− 3.07	6.47E−06	5.34E−05	0.929	0.899	0.958	0.877	0.901	0.851
hsa-miR-214-5p_478768_mir	− 8.13	2.33E−08	9.85E−07	0.923	0.885	0.96	0.877	0.906	0.844
hsa-miR-20b-5p_477804_mir	− 11.65	3.73E−09	2.65E−07	0.921	0.887	0.956	0.883	0.879	0.887
hsa-miR-551b-3p_478159_mir	− 9.71	2.07E−10	3.68E−08	0.919	0.885	0.953	0.852	0.906	0.79
hsa-miR-107_478254_mir	− 4.70	1.35E−03	3.73E−03	0.917	0.883	0.951	0.919	0.938	0.898
hsa-miR-202-5p_478755_mir	− 7.19	5.11E−08	1.30E−06	0.915	0.876	0.954	0.855	0.867	0.842
hsa-miR-93-5p_478210_mir	− 2.84	3.88E−04	1.30E−03	0.915	0.875	0.954	0.86	0.872	0.847
hsa-miR-483-3p_478122_mir	− 7.01	3.69E−09	2.65E−07	0.913	0.877	0.949	0.837	0.884	0.784
hsa-miR-652-3p_478189_mir	− 2.05	2.63E−05	1.70E−04	0.913	0.882	0.945	0.825	0.831	0.818
hsa-miR-29b-3p_478369_mir	− 3.59	3.71E−06	3.47E−05	0.911	0.877	0.945	0.836	0.834	0.838
hsa-miR-328-3p_478028_mir	− 5.03	2.86E−04	1.01E−03	0.908	0.874	0.941	0.819	0.798	0.842
hsa-miR-25-3p_477994_mir	− 2.37	3.44E−05	1.99E−04	0.908	0.87	0.946	0.865	0.899	0.827
hsa-miR-26a-5p_477995_mir	− 2.30	1.99E−05	1.38E−04	0.904	0.872	0.937	0.796	0.799	0.791
hsa-miR-296-5p_477836_mir	− 6.51	3.42E−05	1.99E−04	0.903	0.865	0.941	0.878	0.796	0.973
hsa-miR-144-3p_477913_mir	− 5.05	3.53E−05	1.99E−04	0.903	0.867	0.938	0.827	0.85	0.801
hsa-miR-769-5p_478203_mir	− 3.98	3.53E−05	1.99E−04	0.903	0.864	0.942	0.899	0.901	0.896
hsa-miR-181a-5p_477857_mir	− 2.13	5.21E−06	4.52E−05	0.902	0.865	0.939	0.843	0.895	0.783
hsa-miR-29a-3p_478587_mir	− 3.12	1.16E−03	3.38E−03	0.901	0.86	0.942	0.878	0.854	0.905
hsa-miR-152-3p_477921_mir	− 3.98	2.17E−05	1.48E−04	0.9	0.866	0.934	0.804	0.791	0.819
hsa-miR-125b-1-3p_478665_mir	− 9.13	1.93E−07	3.42E−06	0.895	0.86	0.931	0.86	0.868	0.851
hsa-miR-30a-3p_478273_mir	− 1.66	9.38E−05	4.27E−04	0.891	0.854	0.928	0.812	0.793	0.834
hsa-miR-449b-5p_479528_mir	− 10.10	2.30E−08	9.85E−07	0.889	0.846	0.932	0.88	0.908	0.847
hsa-miR-219a-5p_477980_mir	− 6.58	5.10E−07	7.54E−06	0.889	0.852	0.926	0.84	0.854	0.824
hsa-miR-125a-5p_477884_mir	− 1.40	1.48E−03	4.02E−03	0.888	0.846	0.93	0.827	0.84	0.813

Table 2 (continued)

miRNA	LogFC	p-value	Adj. p-value	AUC	AUC 95% CI_lower	AUC 95% CI_upper	Accuracy	Sensitivity	Specificity
hsa-miR-374b-3p_479421_mir	− 4.64	2.15E−06	2.24E−05	0.887	0.845	0.929	0.8	0.791	0.81
hsa-miR-101-3p_477863_mir	− 4.19	6.98E−05	3.39E−04	0.886	0.845	0.927	0.832	0.896	0.759
hsa-miR-452-5p_478109_mir	− 3.88	2.89E−05	1.80E−04	0.886	0.852	0.92	0.751	0.737	0.766
hsa-miR-193a-3p_478306_mir	− 2.46	2.37E−03	5.97E−03	0.884	0.841	0.928	0.869	0.899	0.835
hsa-miR-148a-3p_477814_mir	− 1.48	1.24E−03	3.52E−03	0.884	0.845	0.923	0.822	0.818	0.826
hsa-miR-133a-3p_478511_mir	− 6.89	1.67E−04	6.73E−04	0.883	0.843	0.922	0.827	0.853	0.797
hsa-miR-675-5p_478196_mir	− 4.22	5.46E−06	4.61E−05	0.883	0.845	0.92	0.766	0.721	0.817
hsa-miR-34a-5p_478048_mir	− 2.04	3.41E−05	1.99E−04	0.882	0.841	0.922	0.823	0.801	0.848
hsa-miR-582-5p_478166_mir	− 8.33	3.37E−07	5.20E−06	0.881	0.842	0.92	0.84	0.767	0.923
hsa-miR-2110_477971_mir	− 4.46	2.45E−06	2.48E−05	0.879	0.837	0.92	0.782	0.794	0.769
hsa-miR-185-5p_477939_mir	− 2.69	1.09E−02	2.18E−02	0.879	0.836	0.921	0.828	0.872	0.777
hsa-miR-144-5p_477914_mir	− 11.46	4.47E−08	1.30E−06	0.877	0.833	0.921	0.873	0.944	0.791
hsa-miR-199a-5p_478231_mir	− 7.83	1.24E−04	5.31E−04	0.877	0.831	0.923	0.772	0.712	0.84
hsa-miR-361-5p_478056_mir	− 1.48	9.56E−04	2.83E−03	0.877	0.837	0.918	0.821	0.86	0.778
hsa-miR-195-5p_477957_mir	− 2.80	2.19E−04	8.17E−04	0.875	0.832	0.919	0.835	0.822	0.849
hsa-miR-136-5p_478307_mir	− 8.01	5.88E−07	8.03E−06	0.873	0.834	0.913	0.828	0.821	0.836
hsa-miR-548d-5p_480870_mir	− 3.80	3.79E−03	8.96E−03	0.873	0.827	0.919	0.755	0.65	0.875
hsa-miR-30b-5p_478007_mir	− 2.55	2.64E−03	6.47E−03	0.873	0.829	0.916	0.774	0.795	0.751
hsa-miR-363-3p_478060_mir	− 8.98	3.58E−05	1.99E−04	0.869	0.825	0.914	0.782	0.776	0.789
hsa-miR-27b-3p_478270_mir	− 2.46	2.87E−02	4.90E−02	0.868	0.828	0.908	0.76	0.815	0.698
hsa-miR-24-3p_477992_mir	− 2.06	4.68E−05	2.40E−04	0.868	0.823	0.913	0.859	0.9	0.813
hsa-miR-499a-5p_478139_mir	− 4.05	4.42E−05	2.34E−04	0.864	0.821	0.908	0.851	0.875	0.824
hsa-miR-15a-5p_477858_mir	− 1.96	3.80E−04	1.28E−03	0.863	0.823	0.903	0.77	0.781	0.758
hsa-miR-31-3p_478012_mir	− 5.27	2.92E−04	1.02E−03	0.86	0.817	0.904	0.799	0.766	0.836
hsa-miR-18a-3p_477944_mir	− 4.70	5.55E−05	2.74E−04	0.859	0.817	0.901	0.761	0.747	0.777
hsa-miR-92a-3p_477827_mir	− 1.48	5.48E−04	1.74E−03	0.859	0.811	0.907	0.813	0.822	0.804
hsa-miR-130b-3p_477840_mir	− 5.57	3.72E−04	1.27E−03	0.858	0.815	0.901	0.748	0.644	0.866
hsa-let-7b-5p_478576_mir	− 1.57	5.76E−04	1.81E−03	0.858	0.814	0.902	0.76	0.758	0.761
hsa-miR-30e-3p_478388_mir	− 4.70	5.51E−03	1.22E−02	0.854	0.81	0.899	0.753	0.628	0.897
hsa-miR-23b-5p_477991_mir	− 3.62	3.36E−05	1.99E−04	0.853	0.81	0.896	0.756	0.758	0.754
hsa-miR-29b-2-5p_478003_mir	− 4.33	2.79E−06	2.75E−05	0.85	0.806	0.894	0.797	0.806	0.786
hsa-miR-30e-5p_479235_mir	− 8.36	6.16E−04	1.92E−03	0.849	0.802	0.896	0.709	0.638	0.791
hsa-miR-200c-3p_478351_mir	− 7.14	2.32E−05	1.53E−04	0.848	0.802	0.894	0.802	0.721	0.894
hsa-miR-1180-3p_477869_mir	− 3.91	4.43E−05	2.34E−04	0.847	0.798	0.896	0.806	0.754	0.866
hsa-miR-190a-5p_478358_mir	− 1.89	1.87E−02	3.48E−02	0.847	0.801	0.892	0.829	0.814	0.847
hsa-miR-151b_477811_mir	− 9.82	2.25E−04	8.31E−04	0.846	0.801	0.892	0.761	0.733	0.792
hsa-miR-505-5p_478957_mir	− 5.86	4.24E−05	2.32E−04	0.846	0.801	0.891	0.796	0.805	0.786
hsa-miR-196b-5p_478585_mir	− 6.46	1.86E−06	2.00E−05	0.845	0.795	0.894	0.767	0.67	0.879
hsa-miR-324-5p_478024_mir	− 1.51	8.13E−03	1.65E−02	0.843	0.797	0.889	0.777	0.754	0.802
hsa-miR-224-5p_477986_mir	− 2.72	1.58E−04	6.45E−04	0.842	0.8	0.883	0.746	0.695	0.804
hsa-miR-139-5p_478312_mir	− 5.12	2.43E−04	8.80E−04	0.839	0.794	0.885	0.727	0.725	0.729
hsa-miR-545-5p_479003_mir	− 5.30	8.42E−06	6.79E−05	0.838	0.79	0.886	0.79	0.79	0.79
hsa-miR-222-3p_477982_mir	− 2.08	7.82E−04	2.37E−03	0.838	0.791	0.884	0.728	0.762	0.689
hsa-miR-340-5p_478042_mir	− 9.05	9.31E−06	7.18E−05	0.836	0.79	0.882	0.803	0.817	0.786
hsa-miR-504-5p_478144_mir	− 3.54	1.86E−04	7.18E−04	0.836	0.787	0.886	0.778	0.729	0.833
hsa-miR-106a-5p_478225_mir	− 10.76	4.30E−08	1.30E−06	0.834	0.784	0.883	0.816	0.924	0.693
hsa-miR-1271-5p_478674_mir	− 9.10	3.87E−06	3.52E−05	0.834	0.785	0.883	0.823	0.9	0.734
hsa-miR-125b-2-3p_478666_mir	− 6.80	9.11E−06	7.18E−05	0.834	0.782	0.886	0.804	0.866	0.735
hsa-miR-339-3p_478325_mir	− 3.60	6.56E−03	1.39E−02	0.834	0.782	0.886	0.778	0.692	0.876

Table 2 (continued)

miRNA	LogFC	p-value	Adj. p-value	AUC	AUC 95% CI_lower	AUC 95% CI_upper	Accuracy	Sensitivity	Specificity
hsa-miR-483-5p_478432_mir	− 6.45	5.42E−07	7.69E−06	0.832	0.783	0.882	0.785	0.871	0.687
hsa-miR-584-5p_478167_mir	− 10.36	6.40E−07	8.25E−06	0.831	0.781	0.882	0.803	0.906	0.685
hsa-miR-17-3p_477932_mir	− 8.43	2.22E−05	1.49E−04	0.831	0.779	0.883	0.805	0.861	0.741
hsa-miR-570-3p_479053_mir	− 4.46	1.11E−04	4.79E−04	0.831	0.785	0.876	0.766	0.697	0.844
hsa-miR-625-5p_479469_mir	− 10.93	4.36E−06	3.87E−05	0.83	0.781	0.88	0.806	0.865	0.738
hsa-miR-196a-5p_478230_mir	− 7.37	1.01E−05	7.64E−05	0.83	0.777	0.883	0.812	0.879	0.735
hsa-miR-7-1-3p_478198_mir	− 7.37	1.00E−04	4.52E−04	0.829	0.783	0.874	0.762	0.646	0.895
hsa-miR-450b-5p_478914_mir	− 9.65	1.03E−07	2.29E−06	0.828	0.775	0.88	0.803	0.906	0.685
hsa-miR-221-5p_478778_mir	− 5.01	3.08E−04	1.06E−03	0.827	0.779	0.875	0.745	0.64	0.865
hsa-miR-128-3p_477892_mir	− 1.32	5.21E−03	1.16E−02	0.823	0.772	0.874	0.743	0.741	0.746
hsa-miR-491-5p_478132_mir	− 3.50	1.88E−03	4.92E−03	0.822	0.774	0.87	0.743	0.703	0.79
hsa-miR-136-3p_477902_mir	− 7.94	2.78E−05	1.76E−04	0.821	0.771	0.871	0.786	0.855	0.707
hsa-miR-101-5p_478620_mir	− 7.44	1.09E−05	8.08E−05	0.819	0.766	0.873	0.812	0.877	0.738
hsa-miR-151a-3p_477919_mir	− 1.93	2.85E−04	1.01E−03	0.819	0.769	0.87	0.803	0.854	0.745
hsa-miR-28-3p_477999_mir	− 2.25	4.30E−03	1.00E−02	0.817	0.771	0.863	0.738	0.728	0.749
hsa-miR-489-3p_478130_mir	− 4.40	1.33E−04	5.62E−04	0.815	0.766	0.865	0.76	0.736	0.787
hsa-miR-106b-3p_477866_mir	− 2.41	6.48E−03	1.39E−02	0.815	0.765	0.866	0.72	0.693	0.751
hsa-miR-324-3p_478023_mir	− 7.87	2.85E−04	1.01E−03	0.814	0.766	0.861	0.761	0.686	0.847
hsa-miR-125a-3p_477883_mir	− 3.24	4.18E−04	1.39E−03	0.811	0.76	0.862	0.765	0.741	0.791
hsa-let-7i-3p_477862_mir	− 6.66	4.69E−04	1.54E−03	0.81	0.759	0.861	0.747	0.665	0.841
hsa-miR-33b-5p_478479_mir	− 5.49	2.06E−03	5.30E−03	0.81	0.758	0.862	0.704	0.626	0.793
hsa-miR-503-5p_478143_mir	− 2.80	6.04E−03	1.31E−02	0.81	0.757	0.863	0.762	0.726	0.805
hsa-miR-301a-3p_477815_mir	− 5.08	1.51E−03	4.05E−03	0.809	0.76	0.858	0.723	0.608	0.856
hsa-miR-330-3p_478030_mir	− 5.72	7.79E−04	2.37E−03	0.805	0.754	0.856	0.761	0.645	0.892
hsa-miR-425-5p_478094_mir	− 1.51	2.66E−02	4.63E−02	0.805	0.757	0.852	0.728	0.714	0.744
hsa-miR-16-2-3p_477931_mir	− 3.32	1.12E−02	2.22E−02	0.804	0.747	0.862	0.78	0.769	0.793
hsa-miR-548k_479374_mir	− 14.17	8.73E−03	1.76E−02	0.801	0.757	0.845	0.718	0.654	0.79
hsa-miR-429_477849_mir	− 2.00	1.76E−02	3.37E−02	0.801	0.747	0.854	0.768	0.807	0.723
hsa-miR-598-3p_478172_mir	− 1.51	4.51E−03	1.04E−02	0.8	0.754	0.847	0.696	0.726	0.661
hsa-miR-887-3p_479189_mir	− 5.41	1.42E−04	5.95E−04	0.799	0.748	0.85	0.737	0.632	0.858
hsa-miR-93-3p_478209_mir	− 4.60	2.28E−04	8.33E−04	0.798	0.745	0.852	0.749	0.702	0.801
hsa-miR-629-5p_478183_mir	− 6.16	1.75E−04	6.90E−04	0.796	0.746	0.846	0.753	0.678	0.839
hsa-miR-21-5p_477975_mir	1.32	7.91E−03	1.61E−02	0.796	0.751	0.842	0.683	0.688	0.677
hsa-miR-140-3p_477908_mir	− 3.42	6.10E−03	1.31E−02	0.793	0.743	0.843	0.734	0.752	0.713
hsa-miR-425-3p_478093_mir	− 5.28	9.90E−04	2.90E−03	0.792	0.74	0.844	0.728	0.588	0.888
hsa-miR-200a-5p_478752_mir	3.05	2.34E−02	4.15E−02	0.792	0.735	0.85	0.713	0.693	0.736
hsa-miR-590-3p_478168_mir	− 4.77	1.27E−03	3.56E−03	0.791	0.739	0.842	0.702	0.637	0.776
hsa-miR-30a-5p_479448_mir	− 8.52	7.71E−04	2.37E−03	0.789	0.738	0.841	0.734	0.726	0.744
hsa-let-7 g-3p_477850_mir	− 6.38	1.74E−04	6.90E−04	0.787	0.733	0.84	0.756	0.608	0.926
hsa-miR-542-3p_478153_mir	− 8.15	2.88E−06	2.76E−05	0.786	0.729	0.842	0.778	0.901	0.638
hsa-miR-31-5p_478015_mir	− 1.78	7.34E−03	1.51E−02	0.786	0.737	0.835	0.676	0.708	0.64
hsa-miR-379-5p_478077_mir	− 5.15	5.46E−04	1.74E−03	0.78	0.724	0.835	0.755	0.773	0.734
hsa-miR-194-5p_477956_mir	− 1.66	1.21E−03	3.48E−03	0.78	0.724	0.836	0.778	0.861	0.683
hsa-miR-34c-5p_478052_mir	− 2.51	4.47E−03	1.04E−02	0.779	0.726	0.832	0.685	0.639	0.738
hsa-miR-576-5p_478165_mir	− 6.85	8.70E−05	4.01E−04	0.778	0.721	0.836	0.774	0.853	0.684
hsa-miR-28-5p_478000_mir	− 5.87	1.45E−03	3.96E−03	0.778	0.726	0.829	0.731	0.64	0.835
hsa-miR-708-3p_479162_mir	− 2.77	2.27E−03	5.75E−03	0.772	0.715	0.828	0.738	0.74	0.736
hsa-miR-505-3p_478145_mir	− 2.12	3.62E−03	8.67E−03	0.771	0.718	0.824	0.758	0.737	0.781
hsa-miR-26b-5p_478418_mir	− 1.01	2.37E−02	4.19E−02	0.768	0.717	0.819	0.692	0.714	0.666

Table 2 (continued)

miRNA	LogFC	p-value	Adj. p-value	AUC	AUC 95% CI_lower	AUC 95% CI_upper	Accuracy	Sensitivity	Specificity
hsa-miR-365a-3p_478065_mir	−4.75	2.67E−02	4.63E−02	0.767	0.713	0.822	0.661	0.495	0.85
hsa-miR-423-3p_478327_mir	−1.63	1.58E−03	4.19E−03	0.765	0.716	0.815	0.65	0.687	0.607
hsa-miR-338-5p_478038_mir	−3.61	2.40E−03	6.01E−03	0.761	0.703	0.819	0.704	0.719	0.687
hsa-miR-210-3p_477970_mir	−1.17	1.67E−02	3.22E−02	0.761	0.708	0.815	0.672	0.698	0.643
hsa-miR-551a_478158_mir	−5.58	1.97E−03	5.10E−03	0.76	0.699	0.822	0.741	0.69	0.8
hsa-miR-889-3p_478208_mir	−8.68	1.14E−05	8.28E−05	0.759	0.698	0.82	0.789	0.922	0.637
hsa-miR-301b-3p_477825_mir	−5.75	1.87E−02	3.48E−02	0.758	0.7	0.816	0.751	0.851	0.637
hsa-miR-590-5p_478367_mir	−3.83	1.63E−02	3.15E−02	0.757	0.703	0.812	0.707	0.59	0.841
hsa-miR-548am-5p_480872_mir	−3.25	1.13E−02	2.22E−02	0.757	0.703	0.81	0.663	0.526	0.82
hsa-miR-187-3p_477941_mir	−4.47	6.97E−03	1.46E−02	0.754	0.701	0.807	0.704	0.644	0.772
hsa-miR-450a-5p_478106_mir	−7.12	1.26E−03	3.56E−03	0.753	0.692	0.814	0.743	0.646	0.853
hsa-miR-376a-5p_478859_mir	−5.94	1.04E−04	4.56E−04	0.75	0.688	0.811	0.738	0.832	0.631
hsa-miR-1296-5p_479451_mir	−4.26	2.49E−03	6.17E−03	0.75	0.695	0.806	0.703	0.719	0.686
hsa-miR-181c-3p_477933_mir	−3.64	9.29E−04	2.77E−03	0.748	0.685	0.811	0.715	0.805	0.613
hsa-miR-1247-5p_477882_mir	−3.36	2.43E−02	4.26E−02	0.748	0.692	0.804	0.698	0.586	0.827
hsa-miR-34a-3p_478047_mir	−2.32	1.77E−02	3.37E−02	0.748	0.694	0.803	0.658	0.727	0.579
hsa-miR-654-3p_479135_mir	−7.56	4.77E−04	1.55E−03	0.74	0.681	0.799	0.76	0.692	0.839
hsa-miR-411-5p_478086_mir	−5.12	7.16E−03	1.49E−02	0.738	0.68	0.796	0.715	0.647	0.793
hsa-miR-181d-5p_479517_mir	−5.67	4.10E−03	9.63E−03	0.733	0.675	0.791	0.686	0.554	0.838
hsa-miR-200b-3p_477963_mir	−3.43	7.26E−03	1.50E−02	0.733	0.674	0.792	0.683	0.609	0.767
hsa-miR-299-3p_478792_mir	−6.51	4.73E−05	2.40E−04	0.732	0.67	0.794	0.748	0.893	0.582
hsa-miR-182-5p_477935_mir	−8.68	8.37E−05	3.91E−04	0.73	0.666	0.795	0.753	0.9	0.585
hsa-miR-410-3p_478085_mir	−5.65	1.22E−03	3.48E−03	0.728	0.67	0.787	0.753	0.771	0.733
hsa-miR-744-5p_478200_mir	−3.55	1.81E−02	3.40E−02	0.725	0.67	0.781	0.657	0.637	0.68
hsa-miR-96-5p_478215_mir	−6.84	1.03E−04	4.56E−04	0.724	0.658	0.79	0.744	0.886	0.583
hsa-miR-133b_480871_mir	−3.18	1.98E−02	3.66E−02	0.724	0.664	0.783	0.686	0.651	0.726
hsa-miR-544a_478156_mir	−5.67	1.37E−03	3.77E−03	0.719	0.66	0.779	0.756	0.81	0.694
hsa-miR-497-3p_478946_mir	−6.92	1.44E−04	5.95E−04	0.718	0.652	0.784	0.753	0.899	0.585
hsa-miR-331-3p_478323_mir	−5.93	3.27E−03	7.90E−03	0.715	0.656	0.774	0.761	0.775	0.744
hsa-let-7f-2-3p_477843_mir	−3.26	1.19E−02	2.33E−02	0.715	0.655	0.776	0.682	0.567	0.814
hsa-miR-195-3p_478744_mir	−4.54	6.67E−03	1.40E−02	0.712	0.652	0.773	0.717	0.712	0.723
hsa-miR-378a-5p_478076_mir	−5.30	2.12E−03	5.41E−03	0.71	0.65	0.769	0.723	0.584	0.882
hsa-miR-1-3p_477820_mir	−6.27	6.10E−03	1.31E−02	0.706	0.644	0.768	0.732	0.778	0.679
hsa-miR-615-3p_478175_mir	−3.14	4.99E−03	1.13E−02	0.706	0.643	0.769	0.696	0.652	0.746
hsa-miR-545-3p_479002_mir	−2.99	1.99E−02	3.66E−02	0.704	0.64	0.767	0.675	0.678	0.672
hsa-miR-548a-3p_478157_mir	−3.75	4.83E−03	1.10E−02	0.703	0.641	0.765	0.716	0.572	0.881
hsa-miR-1248_478653_mir	−2.64	1.08E−02	2.17E−02	0.702	0.642	0.763	0.707	0.764	0.643
hsa-miR-381-3p_477816_mir	−5.10	5.97E−03	1.31E−02	0.701	0.639	0.763	0.734	0.727	0.742
hsa-miR-627-5p_478427_mir	−4.94	5.01E−04	1.62E−03	0.701	0.632	0.77	0.735	0.863	0.589
hsa-miR-1301-3p_477897_mir	−5.30	5.52E−03	1.22E−02	0.696	0.638	0.754	0.711	0.596	0.843
hsa-miR-486-3p_478422_mir	−6.30	1.86E−04	7.18E−04	0.692	0.626	0.758	0.725	0.891	0.535
hsa-miR-200a-3p_478490_mir	−4.37	1.79E−02	3.39E−02	0.691	0.626	0.756	0.719	0.792	0.635
hsa-miR-15a-3p_477928_mir	−3.53	6.65E−03	1.40E−02	0.688	0.62	0.755	0.703	0.808	0.584
hsa-miR-548 g-3p_479020_mir	−2.12	2.25E−02	4.04E−02	0.687	0.625	0.75	0.691	0.759	0.613
hsa-miR-432-5p_478101_mir	−3.66	2.70E−02	4.65E−02	0.683	0.621	0.746	0.691	0.724	0.652
hsa-miR-15b-5p_478313_mir	−6.53	2.89E−02	4.90E−02	0.682	0.619	0.745	0.666	0.692	0.637
hsa-miR-132-5p_478705_mir	−6.01	3.23E−03	7.86E−03	0.68	0.614	0.745	0.734	0.867	0.582
hsa-let-7e-5p_478579_mir	−5.40	2.04E−02	3.72E−02	0.679	0.614	0.744	0.655	0.556	0.769
hsa-miR-485-5p_478126_mir	−2.09	2.46E−02	4.30E−02	0.678	0.613	0.743	0.688	0.734	0.634

Table 2 (continued)

miRNA	LogFC	p-value	Adj. p-value	AUC	AUC 95% CI_lower	AUC 95% CI_upper	Accuracy	Sensitivity	Specificity
hsa-miR-299-5p_478793_mir	−5.05	8.70E−04	2.62E−03	0.672	0.603	0.741	0.702	0.858	0.524
hsa-miR-16-1-3p_478727_mir	−4.44	1.55E−03	4.13E−03	0.669	0.603	0.735	0.699	0.841	0.537
hsa-miR-215-5p_478516_mir	−7.23	8.19E−05	3.88E−04	0.668	0.601	0.735	0.718	0.896	0.514
hsa-miR-103a-2-5p_477864_mir	−4.32	2.05E−02	3.73E−02	0.664	0.603	0.724	0.664	0.529	0.818
hsa-miR-29a-5p_478002_mir	−6.48	2.54E−03	6.26E−03	0.643	0.568	0.717	0.723	0.896	0.524
hsa-miR-874-3p_478205_mir	−2.80	2.77E−02	4.74E−02	0.638	0.575	0.701	0.617	0.524	0.722
hsa-miR-502-5p_478954_mir	−4.03	2.18E−02	3.93E−02	0.637	0.573	0.701	0.662	0.53	0.813
hsa-miR-542-5p_478337_mir	−5.28	3.71E−03	8.83E−03	0.633	0.564	0.702	0.68	0.855	0.481
hsa-miR-362-3p_478058_mir	−5.81	1.73E−03	4.56E−03	0.632	0.564	0.701	0.69	0.879	0.475
hsa-miR-431-3p_478888_mir	−4.55	4.63E−03	1.06E−02	0.624	0.552	0.696	0.678	0.853	0.478
hsa-miR-192-3p_478741_mir	−4.08	5.15E−03	1.16E−02	0.61	0.535	0.685	0.674	0.85	0.474
hsa-miR-589-5p_479073_mir	−2.81	2.28E−02	4.07E−02	0.582	0.512	0.652	0.633	0.795	0.448
hsa-miR-888-5p_479192_mir	−3.79	2.06E−02	3.73E−02	0.559	0.489	0.63	0.656	0.861	0.422
hsa-miR-15b-3p_477929_mir	−3.73	1.76E−02	3.37E−02	0.529	0.459	0.599	0.629	0.86	0.365
hsa-miR-651-5p_479131_mir	−3.94	2.90E−02	4.90E−02	0.523	0.448	0.599	0.638	0.87	0.372

Log fold-change expression, p-value, adjusted p-value, AUC values, accuracy, sensitivity, specificity, and 95% of confidence intervals of the 210 dysregulated miRNAs

CI confidence of interval

profile of serum samples from CRC patients and healthy controls and identified 16 miRNA that were expressed in a significantly higher levels among CRC patients. Of these, 7 miRNAs (let-7a, miR-1229, miR-1246, miR-150, miR-21, miR-223, and miR-23a) were suggested as promising diagnostic biomarkers of CRC with an AUC between 0.67 and 0.95. More recently, the serum exosomal miRNA-19a was found to be upregulated in the serum of CRC patients compared to healthy volunteers, but also was associated with poor prognosis [19]. Finally, Zhao et al. [20], demonstrated that the exosomal miRNA-21 expression is associated with the early diagnosis of CRC. Although plasma and serum have reported promising biomarkers for CRC diagnosis, other approaches as it is the use of proximal bodily fluids as a source of biomarkers have aroused the attention of the biomarker research community. Proximal bodily fluids, such as urine for prostate cancer [21], or uterine fluid for endometrial cancer [22] have demonstrated that this type of fluids offers an improved representation of the molecular alterations that takes place in the tumor. The peritoneal lavage is a proximal fluid with an unexploded value in biomarker research for cancers originating within the peritoneal cavity. Tokuhisa et al. [23] showed that EV-associated miRNAs can be consistently extracted from this bodily fluid and that miRNAs expression profiles can indicate the status of peritoneum in gastric cancer patients.

To the best of our knowledge, our study is the first to report the value of this proximal fluid for the identification of miRNAs associated to EVs in CRC. Importantly, this study unveiled the promising use of the top-10 miRNA dysregulated (miRNA-199b-5p, miRNA-150-5p, miRNA-29c-5p, miRNA-218-5p, miRNA-99a-3p, miRNA-383-5p, miRNA-199a-3p, miRNA-193a-5p, miRNA-10b-5p and miRNA-181c-5p) as diagnostic biomarkers, all showing the AUC value higher than 0.95. Those biomarkers should be validated as well as combined in order to increase the already excellent accuracy of individual miRNAs. However, this should be done

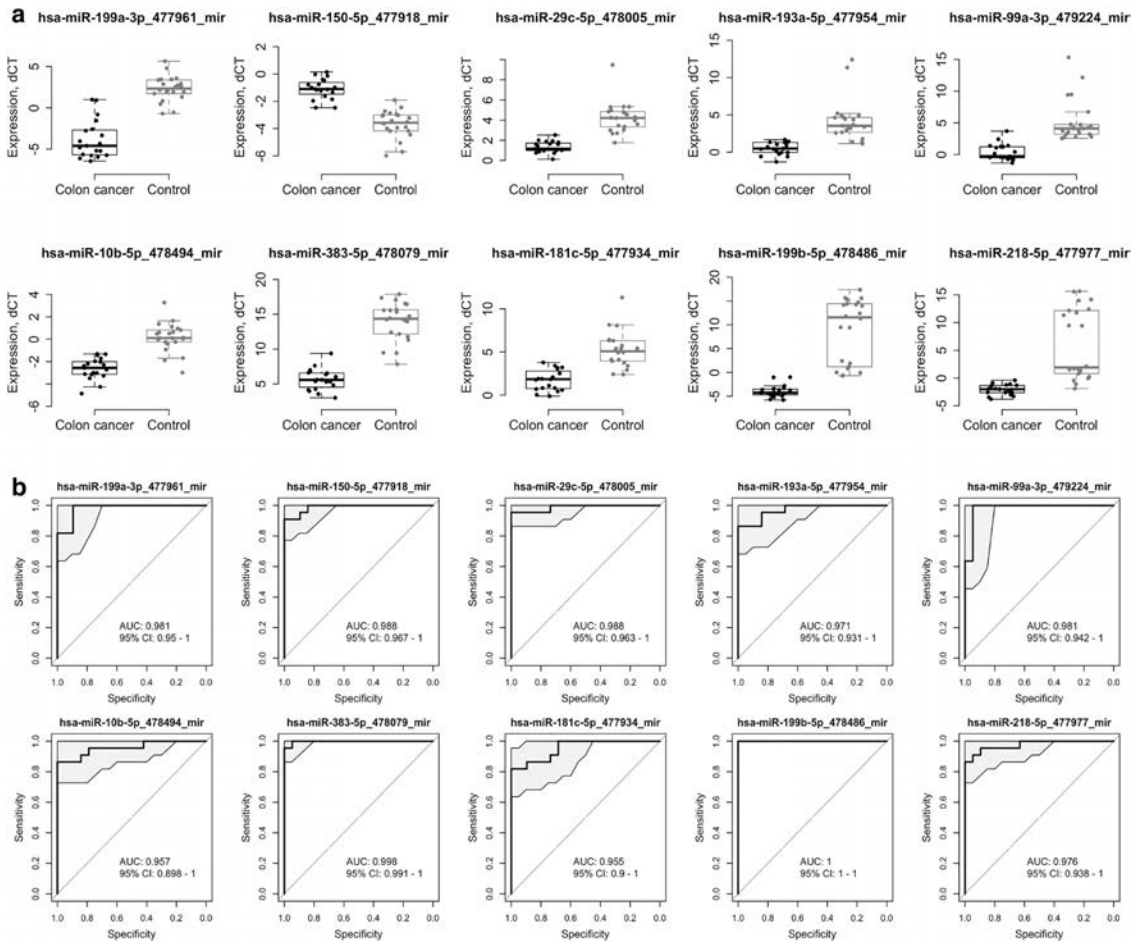

Fig. 1 Diagnostic performance of the top-10 differentially expressed miRNAs. **a** Relative dCT values of top differentially expressed miRNAs in patients with CRC (n = 19) compared to control patients (n = 22). **p < 0.05. **b** ROC-curves and AUC-scores the top-10 differentially expressed miRNAs

Table 3 Published studies of the top-10 miRNAs dysregulated in CRC patients

	Tissue samples	Other type of samples
miR-199b-5p	Not previously reported	Not previously reported
miR-150-5p	Upregulated: [24, 25]	Downregulated: serum [28]
	Downregulated: [26, 27]	
miR-29c-5p	Not previously reported	Not previously reported
miR-218-5p	Upregulated: [29]	Not previously reported
	Downregulated: [30, 31]	
miR-99a-3p	Not previously reported	Not previously reported
miR-383-5p	Downregulated: [32]	Not previously reported
miR-199a-3p	Upregulated: [26]	Upregulated in stool [33, 34]
miR-193a-5p	Downregulated in CRC cell lines [35]	Not previously reported
miR-10b-5p	Downregulated: [30, 36, 37]	Not previously reported
miR-181c-5p	Upregulated: [38]	Not previously reported

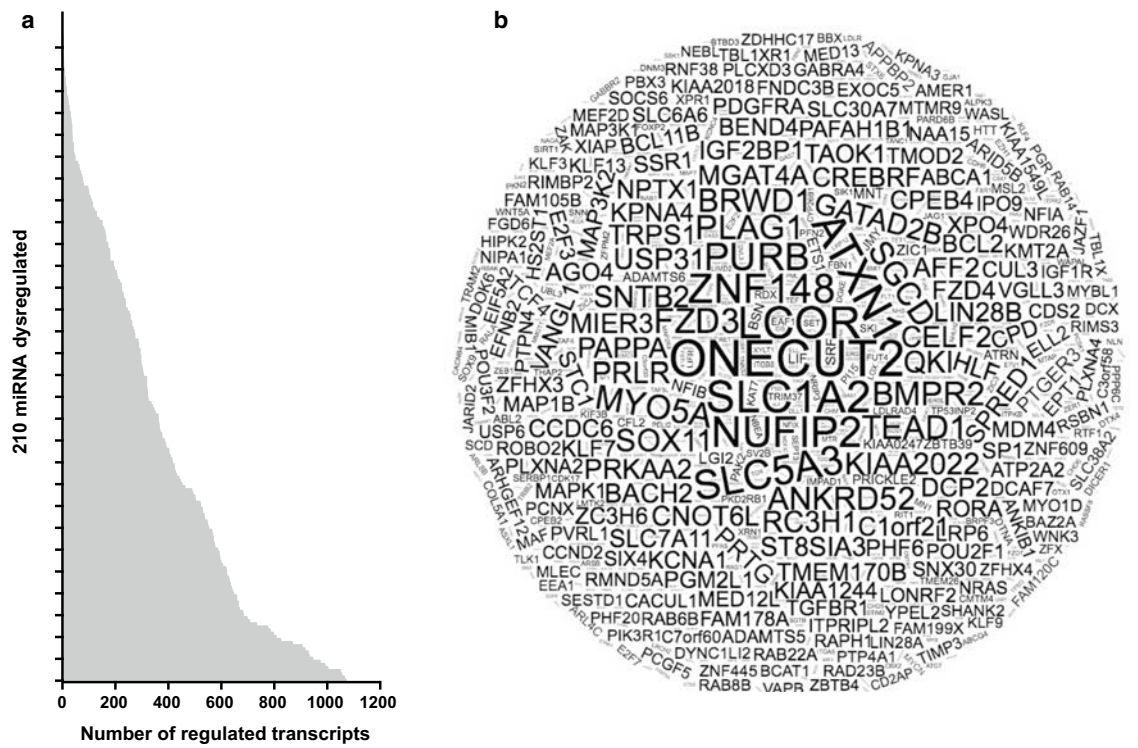

Fig. 2 Prediction of miRNA target transcripts. **a** Boxplot of the number of transcripts regulated by the 210 dysregulated miRNAs. **b** Graphical representation in which the transcript ID is represented with a different font size accordingly to the times that is predicted to be regulated by the different miRNAs, i.e. the most frequently regulated transcripts are shown in a larger font size

in an independent study including a larger cohort of patients. Moreover, further analysis should be performed to elucidate the prognostic value of the detection of the different types of miRNAs in EVs isolated from peritoneal lavages.

Conclusions

In this study, we have demonstrated that use of EV-associated miRNA of ascitic liquid from control patients and peritoneal lavages from CRC patients are an untapped source of biomarkers. Specifically, we identified miRNA-199b-5p, miRNA-150-5p, miRNA-29c-5p, miRNA-218-5p, miRNA-99a-3p, miRNA-383-5p, miRNA-199a-3p, miRNA-193a-5p, miRNA-10b-5p and miRNA-181c-5p as promising biomarkers of CRC diagnosis with the AUC value higher than 0.95.

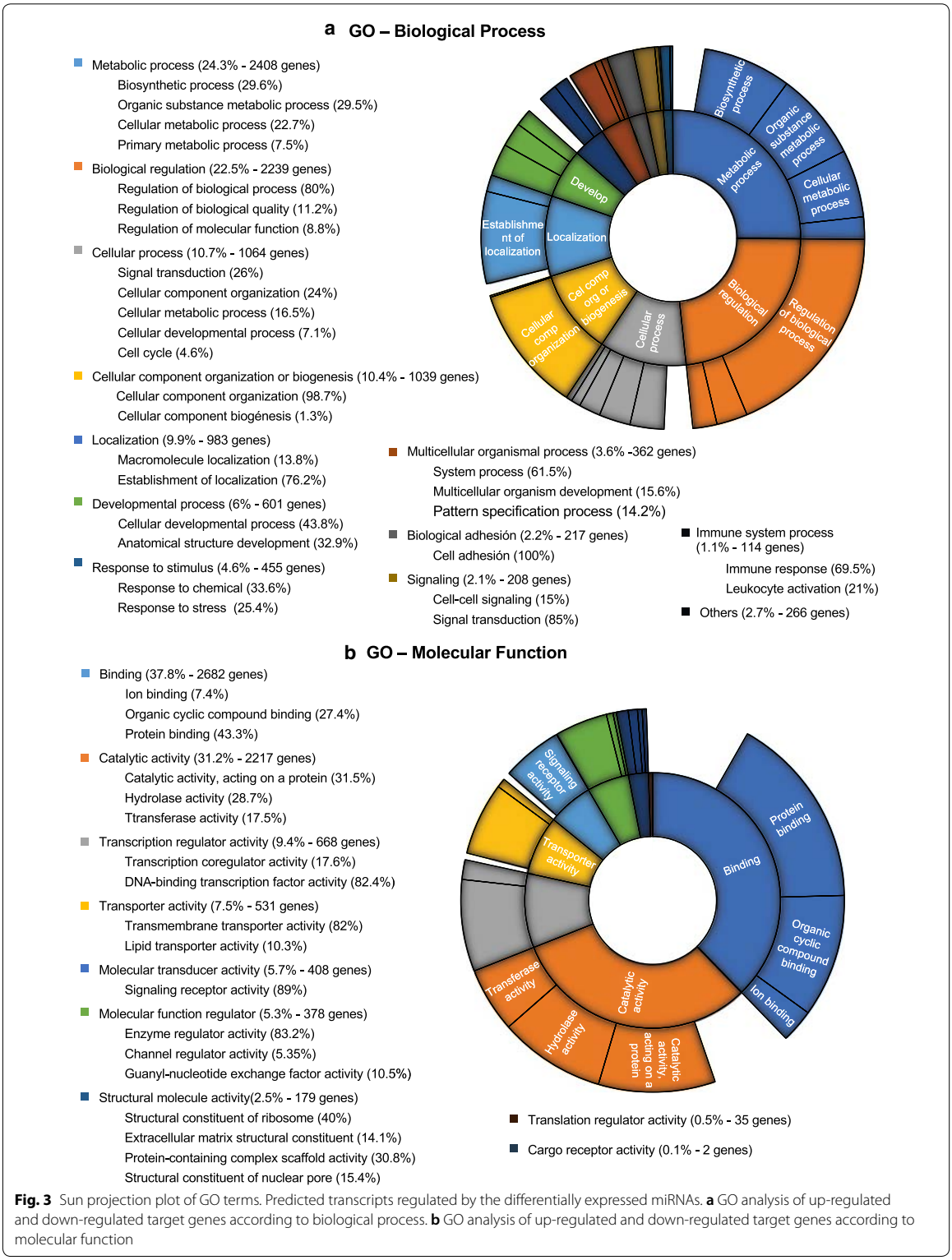

Fig. 3 Sun projection plot of GO terms. Predicted transcripts regulated by the differentially expressed miRNAs. **a** GO analysis of up-regulated and down-regulated target genes according to biological process. **b** GO analysis of up-regulated and down-regulated target genes according to molecular function

Additional files

Additional file 1: Table S1. Clinicopathological characteristics of all patients.

Additional file 2: Figure S1. Workflow. Workflow of the study design.

Additional file 3: Figure S2. EVs characterization. (**A**) Box-plot representing the average mode of EVs isolated from the peritoneal lavage and ascitic fluid of CRC and control patients, respectively (Mean ± SD); measured by Nanoparticle Tracking Analysis. (**B**) Size distribution and concentration of isolated EVs of a peritoneal lavage of a CRC patient (left) and a ascitic fluid of a control patient (right), measured by Nanoparticle Tracking Analysis.

Abbreviations

CRC: colorectal cancer; EVs: extracellular vesicles; RT: reverse transcription; Ct: cycle threshold; FDR: False Discovery Rate; AUC: area under the ROC curve; GO: Gene Ontology; BP: biological process; MF: molecular function; 3′-UTR: 3′-untranslated region.

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Authors' contributions

Study conception and design: XMG, EC. Sample and data collection: SG, MRM, JT, JMP, AGM, JES, MR. Acquisition of data: BRC, CM, SB, EG. Analysis and interpretation of data: SB, JP, CM, BRC, JMFP, EG, XMG, EC. Drafting of manuscript: BRC, CM. Critical revision: CM, JMFP, EG, JMP, JP, XMG, EC. All authors read and approved the final manuscript.

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Availability of data and materials

All bioinformatics analysis was performed with the BioConductor (version 3.7) [10] project in the R statistical environment (version 3.5.0).

Ethics approval and consent to participate

Participants in the study attended to the Hospital Arnau de Vilanova in Lleida, Spain. The Clinical Research Ethics Committee of the hospital approved the study and all the participating patients provided a signed informed consent.

Consent for publication

All authors consent for publication.

Competing interests

The authors declare that they have no competing interests.

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Publisher's Note

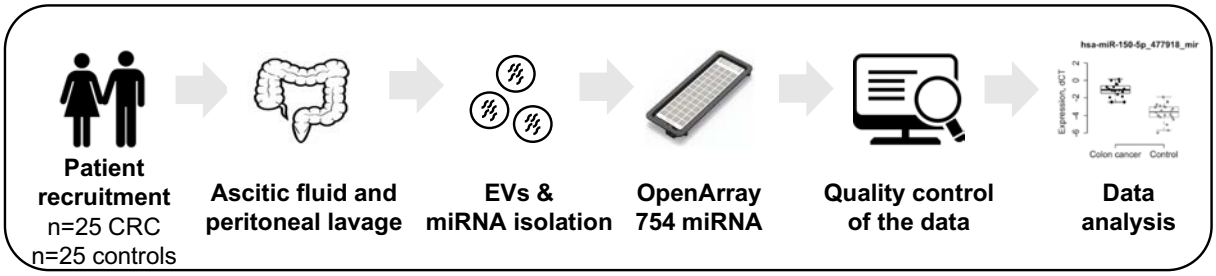
Additional Table S1. Clinicopathological characteristics of all patients.

Patient	Pathology	Age	Gender	Histological diagnostic	Stage	Metastasis	Primary tumor localization
1	Hepatic cirrhosis	74	Male				
2	Hepatic cirrhosis	65	Male				
3	Hepatic cirrhosis	67	Male				
4	Hepatic cirrhosis	60	Male				
5	Hepatic cirrhosis	56	Male				
6	Hepatic cirrhosis	50	Male				
7	Hepatic cirrhosis	54	Male				
8	Heart failure	58	Male				
9	Hepatic cirrhosis	65	Male				
10	Hepatic cirrhosis	63	Male				
11	Hepatic cirrhosis	65	Male				
12	Hepatic cirrhosis	74	Female				
13	Hepatic cirrhosis	62	Male				
14	Hepatic hydrothorax	68	Male				
15	Hepatic cirrhosis	52	Female				
16	Hepatic cirrhosis	78	Female				
17	Hepatic cirrhosis	60	Male				
18	Hepatic cirrhosis	60	Male				
19	Hepatic cirrhosis	51	Male				
20	Hepatic cirrhosis	72	Female				
21	Hepatic cirrhosis	54	Female				
22	Hepatic cirrhosis	90	Male				
23	Hepatic cirrhosis	73	Male				
24	Hepatic cirrhosis	73	Male				
25	Hepatic cirrhosis	70	Male				
26	Colon Cancer	71	Female	ADC Low grade	T2 N0 Mx	No	Ascending Colon
27	Colon Cancer	70	Female	ADC Low grade	T3a N0 Mx	No	Ascending Colon
28	Colon Cancer	66	Male	ADC Low grade	T2 N2a Mx	No	Sigmoid Colon
29	Colon Cancer	65	Female	ADC COM-CRIB	T4 N2a Mx	No	Sigmoid Colon
30	Colon Cancer	78	Female	ADC Low grade	T4a N0 Mx	No	Ascending Colon
31	Colon Cancer	82	Female	ADC Low grade	T2 N0 Mx	No	Transverse Colon
32	Colon Cancer	88	Male	ADC Low grade	T2 N1a Mx	No	Sigmoid Colon
33	Colon Cancer	69	Female	ADC Low grade	Tis N0 M0	No	Ascending Colon
34	Colon Cancer	69	Male	ADC Low grade	T3b N1b Mx	No	Ileocolic Anastomosis
35	Colon Cancer	81	Female	ADC Mucinous	T3d N0 Mx	No	Sigmoid Colon
36	Colon Cancer	69	Male	ADC Micropapillary	T3a N0 Mx	No	Transverse Colon
37	Colon Cancer	75	Male	ADC Low grade	T3a N1 Mx	No	Sigmoid Colon
38	Colon Cancer	51	Female	ADC Low grade	Tis N0 M0	No	Ascending Colon
39	Colon Cancer	74	Male	ADC Low grade	T3a N1b Mx	No	Ascending Colon
40	Colon Cancer	74	Male	ADC Low grade	T2 N0 Mx	No	Ascending Colon
41	Colon Cancer	78	Female	ADC Low grade	T3b N0 Mx	No	Ascending Colon
42	Colon Cancer	74	Female	ADC Low grade	T4a N2b Mx	No	Ascending Colon
43	Colon Cancer	88	Female	ADC Low grade	T3b N0 Mx	No	Descending Colon
44	Colon Cancer	50	Female	ADC Mucinous	T4b N0 Mx	No	Ascending Colon
45	Colon Cancer	84	Male	ADC Low grade	T3 N1 Mx	No	Ascending Colon
46	Colon Cancer	70	Male	ADC Mucinous	T3c N1b Mx	No	Ascending Colon
47	Colon Cancer	74	Male	ADC Low grade	T4a N1b Mx	No	Ascending Colon
48	Colon Cancer	80	Male	ADC COM-CRIB	T3b N1a Mx	No	Ascending Colon
49	Colon Cancer	59	Male	ADC Low grade	T1 N0 Mx	No	Descending Colon
50	Colon Cancer	83	Male	ADC Low grade	T3a N0 Mx	No	Ascending Colon

* ADC: adenocarcinoma
* ADC COM-CRIB: cribiform comedo-type
* G1, G2 and G3: Grade 1, 2 and 3
* N0, N1 and N2: adenopaties afection
* M0: no metastasis
* Mx: no metastasis were seen by the pathologist
* Tis: in situ

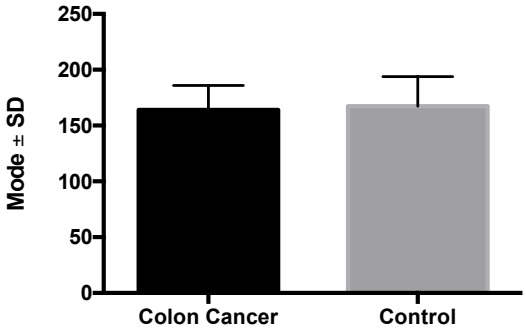
Clinical characteristics of the total cohort of patients recruited in the study.

Additional file 2: Figure S1



Additional file 3: Figure S2

A



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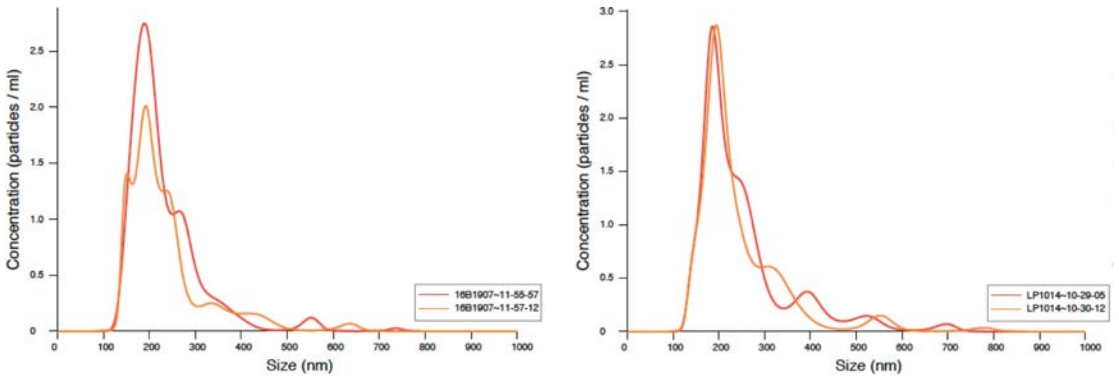

CHAPTER III

EV-associated miRNAs from Pleural Lavage as Potential Diagnostic Biomarkers in Lung Cancer

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EV-associated miRNAs from pleural lavage as potential diagnostic biomarkers in lung cancer

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ABSTRACT

Lung cancer is the leading cause of cancer-related deaths among men and women in the world, accounting for the 25% of cancer mortality. Early diagnosis is an unmet clinical issue. In this work, we focused to develop a novel approach to identify highly sensitive and specific biomarkers by investigating the use of extracellular vesicles (EVs) isolated from the pleural lavage, a proximal fluid in lung cancer patients, as a source of potential biomarkers. We isolated EVs by ultracentrifuge method from 25 control pleural fluids and 21 pleural lavages from lung cancer patients. Analysis of the expression of EV-associated miRNAs was performed using Taqman OpenArray technology through which we could detect 288 out of the 754 miRNAs that were contained in the OpenArray. The differential expression analysis yielded a list of 14 miRNAs that were significantly dysregulated (adj. p-value < 0.05 and logFC lower or higher than 3). Using Machine Learning approach we discovered the lung cancer diagnostic biomarkers; miRNA-1-3p, miRNA-144-5p and miRNA-150-5p were found to be the best by accuracy. Accordance with our finding, these miRNAs have been related to cancer processes in previous studies. This results opens the avenue to the use of EV-associated miRNA of pleural fluids and lavages as an untapped source of biomarkers, and specifically, identifies miRNA-1-3p, miRNA-144-5p and miRNA 150-5p as promising biomarkers of lung cancer diagnosis.

INTRODUCTION

Lung cancer (LC) is the leading cause of cancer-related deaths among men and women in the world, accounting for the 25% of cancer mortality [1]. There are two major forms of LC: non-small cell lung cancer (NSCLC) which is the most common type of LC and include 80% of the cases, and small cell lung cancer (SCLC) [2]. The overall 5-year survival rate of LC is less than 20% mainly due to late diagnosis, whereas patients with tumors diagnosed at early stages have 5-year survival rates of approximately 60%. Diagnosis at early stages of the disease is limited by the fact that LC symptoms occur late in the disease, and current diagnosis rely on the identification of malignant cells from a tissue biopsy [3]. Development of a minimally-invasive diagnosis, based on the identification of sensitive biomarkers in liquid biopsies, could therefore have tremendous impact in decreasing mortality rates with timely therapeutic interventions and disease management.

Several studies have shown the suitability of pleural lavage cytology in early stage surgically resected NSCLC. In these patients, pleural lavage is performed at the beginning of the surgical procedure. Detection of tumor cells in pleural lavage of patients with early stage NSCLC has shown to be associated with shorter overall survival [4]. DNA obtained from pleural lavage material has proven to be appropriate to detect EGFR mutations, even in cases in which tumor cells were not microscopically detected in the lavage [5]. To the best of our knowledge, detection of microRNAs (miRNAs) has not been attempted in this type of material.

MiRNAs are a highly conserved family of small, non-coding RNAs, 19-24 nucleotides in length. They negatively regulate the expression of multiple genes either by including translational silencing or by causing the degradation of messenger RNAs (mRNAs) of the targeted gene, via incomplete base-pairing to a complementary sequence in the 3'-untranslated region (UTR) [6]. MiRNAs are involved in various biologic processes, including cell proliferation, differentiation, death, stress resistances, and fat metabolism; and the aberrant expression of miRNAs has been reported in different diseases and pathological processes including human cancer [2].

miRNAs are detected in tumor tissues but also in body fluids, including extracellular vesicles (EVs). EVs are 20-200 nm round membrane vesicles released by multivesicular bodies fusing with the cell membrane. Their principal function is to participate in the intercellular communication and because of their content in bioactive material such proteins, metabolites, RNA and miRNAs, EVs have been considered an important source of biomarkers for the scientific community. This material is well-protected owing to the EVs lipid bilayer membrane, even if EVs are extracted from circulating or proximal body fluids [7].

To date, several studies have shown the promising role of exosomal miRNAs as diagnostic biomarkers of LC in plasma [8, 9]. Rabinowits et al. in 2009 identified a profile of 12 miRNAs which were increased in both tissue and circulating exosomes of NSCLC patients compared to controls, demonstrating that exosomal miRNA can accurately reflect the tumor profile in the absence of tumor tissue [10]. More recently, Giallombardo et al. [11] unveiled 8 miRNAs that were deregulated in NSCLC comparing to healthy donors and, Jin et al. developed a miRNA profile of 4 miRNAs that exhibited sensitivity of 80.25% and specificity of 92.31% with an AUC value of 0.899 for diagnosing 43 NSCLC patients over 60 controls [12]. Nevertheless, any of those biomarkers have reached the clinical practice, probably due to lack of validation.

New approaches focusing on proximal fluids, i.e. fluids in direct or close contact with the tumor, might provide higher sensitivity and specificity to diagnose LC. Herein, we investigated the use of EVs isolated from the pleural lavage, a proximal fluid in LC patients, as a source of potential diagnostic biomarkers. We conducted miRNA-profiling of EVs isolated from pleural lavages from surgical LC patients, specifically from adenocarcinoma lung cancer (ADC) and lung squamous carcinoma (LUSC) patients, and we unveiled the most relevant individual miRNAs for diagnosing LC. We used a series of non-cancer patients with pleural effusion as a control. The study was conceived as a proof of concept investigation to demonstrate the feasibility of pleural lavage as a source of EV-associated miRNAs in patients with LC.

RESULTS

We analyzed the miRNA profile of EVs isolated from the pleural fluids and lavages of 46 patients, including 25 control and 21 LC patients. Figure 1 illustrates the workflow that was followed in this study. Quality of EVs isolated from the pleural fluids and lavages was measured by size distribution and concentration by nanoparticle Tracking analysis, immunoblot and electron microscopy (Supplementary Figure S1). miRNAs were extracted from EVs for a systematic miRNA expression analysis using the Taqman OpenArray technology through which we could detect 288 out of the 754 miRNAs that were contained in the OpenArray. The quality of the data included the removal of probes that had a Ct value of 40 in all samples, and the removal of samples in which more than 80% of the probes had a Ct value above 40. Finally, a total of 272 miRNA were kept for the differential expression analysis of 20 control and 14 LC patients (Table 1).

The differential expression analysis between cancer and control cases yielded a list of 14 miRNAs that were significantly dysregulated (adj. p-value < 0.05 and logFC lower or higher than 3). Among those, 5 miRNA were found to be upregulated and 9 were downregulated in LC patients (Table 2; Supplementary Figure S2). In order to evaluate whether differential expression translated into diagnostic power, we perform a predictive analysis with all the differentially expressed miRNAs. The logistic model was repeated 500 times to assess the model reproducibility in a divided cohort of training and validation set following a 2:1 ratio; and then the classification performance was evaluated in the whole cohort (Table 3). The best classifier was miRNA-1-3p, which showed an average accuracy of 0.941 (95% CI: 0.803-0.993), sensitivity of 0.929, specificity of 0.950 and AUC value of 0.914. MiRNA-1-30p presented a 13-fold expression, which was lower in LC patients than in controls (adj. p-value of 1.92e-04). The next best classifiers, miRNA-144-5p and miRNA-150-5p, showed an average AUC values comparable with that of miRNA-1-30p with, however, significantly lower accuracy (0.882 and 0.912, respectively) and sensitivity (0.786 and 0.857) for the same specificity. miRNA-144-

5p presented a 11-fold expression which was also lower in LC patients than in controls (adj. p-value of 1.28e-02) while miRNA-150-5p presented an expression higher in LC patients with a 3-fold expression (adj. p-value of 3.91e-02) (Figure 2).

In order to further understand the tumor biology related to the specific EV-associated miRNA content of LC patients, we performed a bioinformatics study to first, unveil the proteins that are regulated by the differential miRNAs, and then, assess their biological and molecular function. A total of 3,745 proteins were found to be regulated by the differential miRNA, specifically 812 proteins were associated to the 5 overexpressed miRNA whilst 2,933 proteins were controlled by the 9 downregulated miRNA (Table 4). To comprehensively integrate the properties of all target proteins, these were studied using Gene Ontology (GO). The most enriched biological functions in LC EVs were cellular processes (29.3%), mostly including cell communication, cell cycle and cellular component movement; and metabolic processes (21.3%), including primary metabolic process, nitrogen compound metabolic process and biosynthetic process (Figure 3A). In relation to the most altered molecular functions in LC EVs, the Gene Ontology (GO) analysis revealed that many targeted proteins were found to be involved in binding (42.8%), including protein and nucleic acid binding; and in catalytic activity (33%), including hydrolase and transferase activity (Figure 3B).

DISCUSSION

In this study, we analyze the EV-associated miRNA profiles of 25 control pleural fluids and 21 pleural lavages from LC patients by using the Taqman OpenArray technology. The differential expression analysis between the two groups yielded a list of 14 miRNAs that were significantly dysregulated, and among them, the best diagnostic biomarkers were miRNA-1-3p, miRNA-144-5p and miRNA-150-5p with an accuracy to label diagnose LC of 0.941, 0.882 and 0.912, respectively.

In our study, miR-1-3p was found to be downregulated by a 13-fold expression in LC patients compared to controls (adj. p-value of 1.92e-04). This is in accordance to

observations by other groups, in which miR-1-3p was identified as a tumor-suppressed miRNA in different types of cancer such as prostate [13, 14], liver [15] and bladder [16]. MiR-1-3p suppressed proliferation, invasion and migration of bladder cancer cells by up-regulating SFRP1 expression [17]. In LC, Nasser et al showed that miR-1 expression is reduced in LC and inhibits the tumorigenic potential of LC cells by down-regulating oncogenic targets, such as MET and FoxP1 [18].

Also in accordance with our finding that miR-144-3p is 11-fold times downregulated in LC, many studies in several types of cancers have reported that miR-144-3p acted as an antitumor miRNA [19, 20] and, recently, it has been reported that both strands of miR-144-5p and miR-144-3p showed a significantly downregulated expression in renal cell carcinoma (RCC) tissues and that they functioned as tumor suppressors in RCC cells [21] and bladder cancer [22]. In LC, miR-144-5p was found to be downregulated in NSCLC clinical specimens as well as in NSCLC cell lines exposed to radiation suggesting that deregulation of the miR-144-5p plays an important role in NSCLC cell radiosensitivity, thus representing a new potential therapeutic target for NSCLC [23]. A recent study revealed that miR-144-5p and miR-451a inhibited cell proliferation [24].

MiRNA-150-5p was upregulated by a 3-fold expression in LC patients. In other studies of LC, miRNA-150-5p was found to be upregulated in tissue suggesting that miRNA-150-5p may be involved in the pathogenesis of LC as an oncogene [25, 26, 27]. However, studies in other types of cancer, i.e. glioma [28], cholangiocarcinoma (CCA) [29] and colon cancer [30], showed a tumor suppressor role of miRNA-150-5p. In vitro experiments on regulation of CCA found that miR-150-5p overexpression inhibited tumor cell proliferation, migration, and invasion capacity, whereas knockdown of miR-150-5p expression induced tumor cell proliferation, migration, and invasion [29]. In colorectal cancer tissues, decreased miR-150-5p was found to be associated with poor overall survival [31].

In the clinical setting, our study provides the evidence that the use of EV-associated miRNA isolated from pleural fluids and lavages are a potential source of biomarkers

for LC. Most of the studies use plasma as it is the most common, easy-to-handle, accessible liquid biopsy. However, the use of proximal fluids offers an improved representation of the molecular alterations that takes place in the tumor. Hence, although proximal fluids, such as the pleural fluid, may occasionally be more difficult to obtain, they might serve as a powerful tool to identify biomarkers for lung-related diseases. In relation to proximal fluids related to LC, studies performed by Admyre et al. [32] and more recently, Ji Eun Kim et al. [33] highlighted the use of another type of fluid, i.e. the bronchoalveolar lavage (BAL). Although this fluid is obtained in a non-invasive manner, biomarkers identified in BAL might only represent tumors localized within the lung and/or in direct contact with the airway. Nevertheless, pleural lavages are expected to provide biomarkers from tumors localized in different sites, i.e. inside and outside of the lungs. Importantly, our study unveiled the promising use of miRNA-1-3p, miRNA-144-5p and miRNA-150-5p as diagnostic biomarkers. Those biomarkers should be validated as well as combined in order to increase the already excellent accuracy of the individual miRNA. However, this should be done in an independent study including a larger cohort of patients and controls. Interestingly, mesothelioma patients might also be compared to LC patients in future studies. Moreover, further analysis should be performed to elucidate the prognostic value of the detection of the different types of miRNAs in EVs isolated from pleural lavages.

CONCLUSION

In this work, we have demonstrated that use of EV-associated miRNA of pleural fluids and lavages are an untapped source of biomarkers, and specifically, we identified miRNA-1-3p, miRNA-144-5p and miRNA 150-5p as promising biomarkers for LC diagnosis.

METHODS

Patients and pleural fluid and lavages collection. A total of 46 participants were recruited at Hospital Arnau de Vilanova in Lleida, Spain. All the patients participating

signed an informed consent and the study was approved by the Clinical Research Ethics Committee of the hospital. All experiments were performed in accordance with relevant guidelines and regulations of the hospital. Pleural fluids and lavages were extracted from a cohort of 46 patients, corresponding to 25 control patients with benign pleural effusions, and 21 patients with ADC or LUSC, who underwent curative surgery. In control patients, the collection of pleural fluid was performed under local anesthesia (2% mepivacaine) by the introduction of a metallic needle in the pleural cavity through an intercostal space. The pleural fluid was gently aspirated, collected in a 50 mL tube and stored at -80ºC. In LC patients, the pleural lavage was collected, during surgery, after accessing the thoracic cavity and prior to any manipulation of the lung. A total of 100 cc of physiological saline were instilled into the pleural cavity with a 50 cc syringe, mobilizing the patients for its correct distribution of the serum and were extracted with a 50 cc syringe connected to a 14-gauge aspiration needle. A volume ranging from 80 to 90 mL was collected in 50 mL tubes and stored at -80ºC. All fluids were non-hemorrhagic and proved to be exudates. The clinical features of each patient are listed in Supplementary Table S1. The diagnosis of LC was based on cytohistological background, while that of benign pleural effusions relied on well-established clinical criteria.

EVs isolation. EVs were isolated with a differential centrifugation method, following a modification of a previously described EVs isolation protocol [34]. Pleural fluids and lavages were centrifuged by Thermo Scientific Heraeus MultifugeX3R Centrifuge (FiberLite rotor F15-8x-50c) at 300xg during 10 min, followed by a centrifugation step at 2500xg during 20 min and a centrifugation step at 10,000g during 30 min. After, the supernatant was filtered through 0.22 µm filters (Merck Millipore) and the sample obtained was transferred to ultracentrifuge tubes (Beckman Coulter) and filled with PBS. To finish the centrifuged procedure, two consecutive ultracentrifugation steps at 100,000 g were performen on a Thermo Scientific Sorvall WX UltraSeries Centrifuge with an AH-629 rotor during 2 hours each. At the end, the pellet obtained with the EVs was resuspended in 50 µL of PBS. From those, 5 µL were isolated for

nanoparticle tracking analysis (NTA) and quantification, and the rest was frozen at -80ºC with 500 µL of Qiazol for RNA extraction, or with 45 µL of RIPA buffer (5nM EDTA, 150mM NaCl, 1% Triton, 20nM Tris pH8 and 1:200 protein inhibitors) for protein extraction.

Nanoparticle Tracking Analysis. NTA was performed using a_Nanosight LM10 instrument equipped with a 405 nm laser and a Hamamatsu C11440 ORCA-Flash 2.8 camera (Hamamatsu) with Nanoparticle Tracking Analysis (NTA, Malvern Instruments, UK) and data was analyzed with the NTA software 2.3 following the manufacter's instructions. To define the size and concentration of the particles, the samples were diluted appropriately with Milli-Q water (Milli-Q Synthesis, Merck Millipore, Massachusetts, USA) to give counts in the linear range of the instrument. The particles in the laser beam undergo Brownian motion, and a video was recorded for 60 s in triplicate.

Immunoblot. Protein extracts of EVs were obtained by unfrozen the RIPA-containing EVs samples, incubating for 1 h at 4ºC, and sonication. Protein extracts were loaded and separated by a 10% SDS-PAGE and transferred to PVDF membranes. For blocking, membranes were soaked in 5% non-fat dried milk in TBS-Tween20 (0.01%). Proteins were immunodetected using primary antibodies: mouse anti-CD9 (1:250; ref. 555370, BD Biosciences) and mouse anti-TSG101 (1:500; ref. ab83, Abcam). For the incubation with a secondary HRP-coupled antibody (rabbit anti-mouse Immunoglobulins/HRP, 1:2000, ref. P0260, Dako), PVDF membranes were firstly washed and then the incubated was performed. Finally, we revealed using the Immobilon Western Chemiluminiscent HRP Substrate (ref. WBKLS0100; Merck Millipore) and the intensity of the bands was quantified using the Image J software (v. 1.45s).

Electron microscopy. For cryo-electron microscopy, EV preparations were directly adsorbed onto glow-discharged holey carbon grids (QUANTIFOIL, Germany). Grids were blotted at 95% humidity and rapidly plunged into liquid ethane with the aid of a VITROBOT (Maastricht Instruments BV, The Netherlands). Vitrified samples were

imaged at liquid nitrogen temperature using a JEM-2200FS/CR transmission cryo-electron microscope (JEOL, Japan) equipped with a field emission gun and operated at an acceleration voltage of 200 kV.

Total RNA extraction. The total RNA was isolated from the EVs samples containing Qiazol by using the miRNeasy MiniKit (Qiagen) and following the manufacturers' protocol. RNA from EVs was eluted with 30 µL of Nuclease-free water (Ambion) and then were stored at -80°C for their future utilization.

Openarray analysis. miRNA expression was performed using a fixed-content panel containing 754 well-characterized human miRNA sequences from the Sanger miRBase v14 (Catalog number: 4470187, Thermo Fisher Scientific) following the procedure of previous studies of the group [35, 36]. Reverse transcription (RT) was performed on 2 µL RNA using Megaplex™ Primer Pools A and B and the supporting TaqMan® MicroRNA Reverse Transcription Kit as follows: 15 min at 42ºC and 5 min at 85ºC. Then, 5 uL of the resulting cDNA was preamplified prior to real-time PCR analysis using Megaplex™ PreAmp Pools and the TaqMan® PreAmp Master Mix using the following conditions: one single step at 95ºC during 5 min, 20 cycles of a two-steps program (3 sec, 95ºC and 30 sec, 60ºC) followed by a single cycle of 10 min at 99ºC to inactivate the enzyme. The preamplified products were diluted 1:20 in 0.1x TE buffer pH8.0, and mixed in 1:1 with TaqMan® OpenArray® Real-Time PCR Master Mix in the 384-well OpenArray® Sample Loading Plate. TaqMan® OpenArray® MicroRNA Panels were automatically loaded using the AccuFill™ System.

Preprocessing and differential expression analysis. The bioinformatics analysis was performed with the BioConductor (version 3.7) [37] project in the R statistical environment (version 3.5.0) [R Core Team (2015): R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/]. HTqPCR (version 1.34) R package [38] was used to proceded the data. Probes that had a "Cycle threshold" (Ct) value of 40 in all samples were removed. Further samples in which more than 80% of the probes had a Ct value

above 40 were retained. To assure comparability across samples, the Ct values were delta normalized. The average of the probes hsa−miR−324−5p, hsa−miR−128−3p, hsa−miR−24−3p, and hsa−miR−148a−3p were used for normalization of the Ct values. Those probes were selected based on having Ct value of 40 in a maximum of three samples, and the lowest interquartile range across samples. Differential expression analysis was carried out with an empirical Bayes approach on linear models, using the limma (version 3.36) R Package [39]. Results were corrected for multiple testing using the False Discovery Rate (FDR) [40].

Development of predictors. The whole patient cohort was divided into training and validation sets with the 2:1 ratio for predictive analysis,. Calculated (with limma) relative miRNA expression values were used as input variables to a logistic regression model between groups. Each significant (adj. p-value < 0.05) deregulated miRNA was fitted into the logistic regression model to differentiate the LC and the control patient's groups; and the model classification performance was evaluated using the AUC (area under the ROC curve), accuracy, sensitivity and specificity values on the validation set. The procedure of partitioning the dataset into training and validation sets and fitting the logistic model was repeated 500 times to assess the model reproducibility and collect statistics. Finally, AUC values for each selected predictor were calculated in the whole cohort.

Prediction of miRNA target genes and bioinformatics analysis. Predicted miRNAs target genes were obtained using the Predictive Target Module of miRWalk2.0 online software [41] (https://goo.gl/ajG9ja). To improve the accuracy of target gene prediction and reduce the rate of false positives, we considered as valid target genes only those transcripts that were predicted in at least 8 out of the 12 databases (miRWalk, miRanda, MicroT4, miRDB, miRMap, miRBridge, miRNAMap, PICTAR2, RNA22, PITA, TargetScan, and RNAhybrid). To analyze the potential functions of the predicted target genes, we performed a Gene Ontology (GO) functional analysis using the online Panther software [42] (http://www.pantherdb.org/).

Biological process (BP) and molecular function (MF) GO terms were analyzed and plotted.

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AUTHOR CONTRIBUTIONS

Study conception and design: JMP, XMG, EC, AGM, JMFP, JP.
Sample and data collection: MRM, SG, AO, JLR.
Acquisition of data: BRC, CM, EG, SB.
Analysis and interpretation of data: BRC, SB, JP, EC.
Drafting of manuscript: BRC, CM, JP, EC, XMG.
Critical revision: EC, BRC, CM, MRM, SG, AGM, JMP, JMFP, EG, SB, JP, XMG, AO, JLR.

COMPETING INTERESTS

The author(s) declare any competing financial and/or non-financial interests in relation to the work described.

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TABLES

Table - 1

	Lung Cancer	Control
Age		
Median	68	77
Minimum	52	62
Maximum	84	92
Gender		
Female	2	9
Male	12	11
Pathology		
Lung Cancer	14	-
ADC	9	-
LUSC	7	-
Heart failure	-	14
Hepatic hydrothorax	-	2
Post CABG surgery	-	1
Constructive pericarditis	-	1
SVC obstruccion	-	1
Chronic kidney disease	-	1

* ADC: adenocarcinoma
* LUSC: lung squamos carcinoma
* SVC: superior vena cava
* CABG: coronary artery bypass graft

Table - 2

ID	logFC	P-Value	adj.P-Va
hsa-miR-150-5p_477918_mir	**3,65**	**1,87E-03**	**3,91E-02**
hsa-miR-144-5p_477914_mir	**-11,37**	**3,76E-04**	**1,28E-02**
hsa-miR-1-3p_477820_mir	**-13,78**	**1,41E-06**	**1,92E-04**
hsa-miR-584-5p_478167_mir	-9,55	1,90E-08	5,17E-06
hsa-miR-133b_480871_mir	-7,72	1,30E-03	3,52E-02
hsa-miR-451a_478107_mir	-3,29	3,50E-04	1,28E-02
hsa-miR-27a-5p_477998_mir	4,93	8,05E-05	7,30E-03
hsa-miR-21-3p_477973_mir	5,65	1,99E-04	1,28E-02
hsa-miR-199a-5p_478231_mir	-7,73	3,47E-04	1,28E-02
hsa-miR-1249-3p_478654_mir	6,70	5,62E-04	1,70E-02
hsa-miR-485-3p_478125_mir	4,80	1,82E-03	3,91E-02
hsa-miR-20b-5p_477804_mir	-8,18	3,05E-04	1,28E-02
hsa-miR-181c-5p_477934_mir	-3,79	2,06E-03	4,01E-02
hsa-miR-30e-5p_479235_mir	-6,46	1,45E-03	3,59E-02

Table - 3

Logistic model on the 2:1 cohort					
miRNA	**AUC**	**Accuracy**	**95% CI**	**Sensitivity**	**Specificity**
hsa-miR-1-3p_477820_mir	0,923	0,941	[0.936; 0.946]	0,938	0,943
hsa-miR-144-5p_477914_mir	0,925	0,878	[0.872; 0.883]	0,776	0,941
hsa-miR-150-5p_477918_mir	0,937	0,825	[0.818; 0.831]	0,666	0,925

Logistic model on the whole cohort					
miRNA	AUC	Accuracy	95% CI	Sensitivity	Specificity
hsa-miR-1-3p_477820_mir	0,914	0,941	[0.803; 0.993]	0,929	0,95
hsa-miR-150-5p_477918_mir	0,939	0,912	[0.763; 0.981]	0,857	0,95
hsa-miR-144-5p_477914_mir	0,925	0,882	[0.725; 0.967]	0,786	0,95

Table - 4

5 miRNA up-regulated - target 812 unique proteins

miRBASE code	ID	logFC_A_vs_B	# Proteins regulated *
MIMAT0000451	hsa-miR-150-5p_477918_mir	3,65	518
MIMAT0002176	hsa-miR-485-3p_478125_mir	4,80	247
MIMAT0004501	hsa-miR-27a-5p_477998_mir	4,93	33
MIMAT0004494	hsa-miR-21-3p_477973_mir	5,65	55
MIMAT0005901	hsa-miR-1249-3p_478654_mir	6,70	9

9 miRNA down-regulated - 2933 unique proteins

miRBASE code	ID	logFC_A_vs_B	# Proteins regulated *
MIMAT0000416	hsa-miR-1-3p_477820_mir	-13,78	460
MIMAT0004600	hsa-miR-144-5p_477914_mir	-11,37	19
MIMAT0003249	hsa-miR-584-5p_478167_mir	-9,55	269
MIMAT0001413	hsa-miR-20b-5p_477804_mir	-8,18	1060
MIMAT0000231	hsa-miR-199a-5p_478231_mir	-7,73	364
MIMAT0000770	hsa-miR-133b_480871_mir	-7,72	429
MIMAT0000692	hsa-miR-30e-5p_479235_mir	-6,46	662
MIMAT0000258	hsa-miR-181c-5p_477934_mir	-3,79	865
MIMAT0001631	hsa-miR-451a_478107_mir	-3,29	13

FIGURE AND TABLE LEGENDS

Figure 1. Workflow. Workflow of the study design.

Figure 2. Diagnostic performance of the top differentially expressed miRNAs. (A) Relative dCT values of top differentially expressed miRNAs (miRNA-1-3p, miRNA-150-5p, and miRNA-144-5p) in patients with lung cancer (n=14) compared to control patients (n=20). ** $p < 0.05$. (B) ROC-curves and AUC-scores for miRNA-1-3p, miRNA-150-5p, and miRNA-144-5p.

Figure 3. GO terms associated to the predicted proteins regulated by the differentially expressed miRNAs in lung cancer and control patients. (A) GO analysis of up-regulated and down-regulated target genes according to biological process. (B) GO analysis of up-regulated and down-regulated target genes according to molecular function.

Table 1. Clinicopathological characteristics of patients. Clinical characteristics of the final cohort of patients included in the study after data normalization.

Table 2. miRNA transcripts displaying a significant differential expression in patients with lung cancer compared to control patients. Log fold-change expression, p-value and adjusted p-value of the 14 miRNAs significantly dysregulated in EVs from the pleural lavage of lung cancer patients compared to control (adj. p-value < 0.05 and logFC lower or higher than 3).

Table 3. Performance of the top diagnostic miRNA biomarkers. AUC values, accuracy, sensitivity, specificity and 95% of confidence intervals are summarized for those miRNAs which were selected for the highest diagnostic performance on the 2:1 cohort (on top) and the whole cohort (below).

Table 4. Prediction of miRNA target proteins. The proteins regulated by each miRNA was predicted using the Predictive Target Module of miRWalk 2.0 sofware and minimized to those that were found in at least 8 out of 12 databases. The total number of predicted proteins is plotted for each dysregulated miRNA.

DISCUSSION

Cancer is an important health concern worldwide and represents the second most common reason for mortality worldwide after cardiovascular diseases. More than half of the patients diagnosed with cancer around the world succumb to it. Moreover, since the world population aging is increasing as well as the cancer associated lifestyle habits, cancer will promptly turn into the first cause of death in many parts of the world [2, 1].

Among the different types of cancer that exists, endometrial, colorectal and lung cancers are ranged in the top-10 cancers worldwide, both in terms of incidence and mortality.

Endometrial cancer (EC) is the most common gynecological malignancy of the female genital track and the fourth most common cancer in women worldwide with 570,000 new cases and 311,00 deaths estimated in 2018. EC is a gynecological disease that shows a continuous increasing incidence among older, but also younger, patients. There are about 30% of EC patients diagnosed at advanced stages of the disease, presenting a bad prognosis and a drastic decrease in the 5-years survival rate. Colorectal cancer (CRC) is the second leading cause of cancer death worldwide with 881,000 deaths in 2018, and the fourth most commonly diagnosed cancer worldwide with 1,800,00 cases. Although the 5-year survival rate is quite high when patients are diagnosed at initial stages, the rate decreases dramatically to the 14% of survival when patients are diagnosed at advanced stages. Finally, lung cancer (LC) is the leading cause of cancer incidence and mortality worldwide. With 2.1 million new cases and 1.8 million deaths predicted in 2018, the disease has become an epidemic as incidence and deaths rates have risen dramatically over the last decades. The mortality rate is closely parallel to the incidence rate because of the low patient survival. More than half of the patients (57%) are diagnosed at advanced stage when the tumor is not localized and the 5-year survival is about 5%.

Despite the fact that some symptoms are related to these cancers, they are not highly specific as other benign disorders generate a similar symptomatology. The lack of specific symptoms and accurate screening methods are the reasons why **EC, CRC and LC are mostly diagnosed at advanced stages when the survival is compromisingly low. Hence, research towards the development of new tools to improve the clinical management of patients is much needed in order to improve survival in EC, CRC and LC patients.**

To date, treatment-decision making on these three types of cancers has been performed based on clinical and pathological findings. However, this may represent a limitation in order to successfully manage cancer patients since we know that tumors with similar clinical and pathological features can present different molecular profiles, and moreover, genetic information might be different within the same tumor (i.e. intratumor heterogeneity). Thus, it is expected that the inclusion of molecular-based tools in the clinical management of cancer patients will be of great benefit to improve their survival.

In the last years, several studies have been performed to achieve the **identification of novel tumor biomarkers in order to improve the management of EC, CRC and LC patients.** Nevertheless, none of these potential biomarkers have been validated, nor have reached clinical practice still. In most of those studies, tumor tissue biopsy has been the gold standard of cancer subtyping in which the molecular profile of cancers is typically assessed using DNA and/or RNA obtained from a fragment of the primary tumor or a single metastatic lesion (tumor tissue biopsy) [61]. Although tumor tissue biopsy is a good source of biomarkers because presents a higher concentration of the molecular alterations, there are important disadvantages: The most important **is the difficulty and/or invasiveness for the collection of the tissue biopsy,** which hampers its use in many cases. Other limitations include that tissue biopsies may do not represent accurately the heterogeneous tumor tissue,

they are usually taken from the primary tumor and reflect its molecular composition at the time of the sample taken and, additionally the mere analysis of the resected primary tumor alone may provide a misleading information with regard to the characteristics of metastases.

In order to overcome the tissue biopsy limitations, **the study of the tumor material present in body fluids has become an important tool in biomarker research.** The possibility of probing the molecular landscape of solid tumors via a blood draw, with major implications for research and patient care, has attracted remarkable interest among the oncology community and the term 'liquid biopsy' describe this approach. Among liquid biopsies, plasma and serum are the most commonly used. However, in contrast to blood, proximal fluids are in direct contact or close to a certain organ or part of the body and it has been shown that these proximal fluids are highly enriched in molecules derived from diseased tissue and reflect microenvironmental or systemic effects of the disease, turning them into an attractive source of biomarkers. Proximal body fluids, such as urine for prostate cancer [114], or uterine fluid for EC has offered an improved representation of the molecular alterations that takes place in the tumor [75], but research on this type of fluids is quite limited. **Thus, in this thesis we have evaluated the use of peritoneal and pleural lavages, two proximal fluids, for the search of EC, CRC and LC cancers biomarkers. To do this, we have exploited the benefit of using extracellular vesicles.**

We demonstrated that exosomes exist in peritoneal and pleural lavages as well as in ascitic and pleural fluids. We isolated exosomes based on ultracentrifugation method and moreover, we carried out an extensive characterization describing their morphology, size and, in some case, its enrichment in well-known exosome markers and electron microscopy. In all our studies, we noticed that although we followed a protocol for the isolation of exosomes, our population was composed by various types of small membrane vesicles raging from 20 to 200 nm, but also for larger

vesicles, probably microvesicles. This has been described by others, and it is commonly accepted by the scientific community to use the term extracellular vesicles (EVs) in those cases. Thus, in our work, although exosomes were the main focus of our research, and our sample is enriched in exosomes, we have referred to EVs in all our studies.

Our work is the first one that has analyzed the EV-associated miRNAs expression in peritoneal and pleural lavages from EC, CRC and LC patients. Nevertheless, other studies have used the same or similar fluids to investigate other cancer types. Recently, Motohiko Tokuhisa et al [115] showed that EV-associated miRNAs can be consistently extracted from peritoneal lavages and are representative of the existence of gastric cancer. In EC and CRC, peritoneal lavages have been described as proximal body fluids but nowadays; they have not been yet explored to investigate EC and CRC-related biomarkers. On the other hand, in LC, other proximal fluids such as bronchoalveolar fluid have been studied. The first published study on biomarkers research in bronchoalveolar lavage (BAL) was conducted by Admyre et al. [116] and more recently, Ji Eun Kim et al. [117] also published on this topic. Both of them pointed out the potential of BAL as a useful source of biomarkers for diagnosis of early-stage lung adenocarcinoma. BAL is a body fluid obtained in a non-invasive manner from the patient, and it is expected to contain mainly biomarkers from a tumor that is localized within the lungs and in direct contact with the airway but in contrast to pleural lavages, cannot provide biomarkers from tumors localized in different sites, i.e. inside and outside of the lungs.

The main disadvantage of peritoneal and pleural lavages is that their collection entails an invasive procedure. They are obtained just before surgery by introducing and subsequently recovering serum in the peritoneal and pleural cavities. Once the fluid is obtained, surgeon completes surgery by remaining the organ that contain the tumors. Although this is an important limitation to develop diagnostic biomarkers, the

significant molecular information retrieved from these fluids might contain prognostic information. Moreover, the most significant miRNAs identified in this thesis might be explored in other more accessible body fluids to develop a non or minimally invasive tool for early diagnosis of EC, CRC and LCs.

This thesis is divided into three chapters each one of them associated to a specific objective: 1) Identification of EV-associated miRNAs biomarkers in peritoneal lavage to improve management of EC patients; 2) Identification of EV-associated miRNAs biomarkers for CRC in peritoneal lavage; and 3) Identification of EV-associated miRNAs biomarkers for LC in pleural lavage.

In Chapter 1- "Identification of EV-associated miRNAs biomarkers for EC in peritoneal lavage"- we investigated for the first time the miRNA content of EVs isolated from 25 peritoneal lavages from EC patients and 25 ascitic fluids from control patients. As a result of this work, we could confirm that the isolation and enrichment of EVs from peritoneal lavages and ascitic fluids was successful, obtaining a major population of vesicles with a mean mode of 204.3 (measured by Nanosight®). The EV-associated miRNAs were analyzed using the Taqman OpenArray technology and the differential expression analysis yielded 114 miRNAs that were significantly dysregulated in EC patients. Among those, 96 miRNAs were downregulated and 18 miRNAs were upregulated in cancer patients compared to non-cancer patients. An abundance of scientific research has been published regarding the role of miRNAs in EC [118]. Torres et al. published the first study focused on miRNA expression both in tissue and plasma samples of EC patients and a combined signature of 2 miRNAs in plasma samples resulted in 88% sensitivity and 93% specificity, indicating a good diagnostic potential [119]. Despite these findings, they were not applied in the clinical setting [120]. In EC, miRNAs isolated from EVs have been scarcely studied. Akhil et al. evaluated the potential of miRNA content of the urine-derived EVs as diagnostic biomarkers in EC patients [121], and Hanzi Xu et al. isolated EVs from serum samples and identified 209 upregulated and 66 downregulated circRNAs in EVs from serum

of patients with EC compared with those from healthy controls [122]. Despite these previous studies, to the best of our knowledge, our study is the first to report the value of this proximal fluid for the identification of miRNAs associated with EVs in EC. These findings describe the dysregulation of 114 miRNAs, among which miRNA-383-5p, miRNA-10b-5p, miRNA-34c-3p, miRNA-449b-5p, miRNA-34c-5p, miRNA-200b-3p, miRNA-2110 and miRNA-34b-3p are of special interest to further investigate their promising use as EC biomarkers, as they demonstrated a high classification potential with an AUC value higher than 0.90.

In **Chapter 2- "Identification of EV-associated miRNAs biomarkers for CRC in peritoneal lavage"-** we aimed to identify for the first time, the miRNA content of EVs isolated from peritoneal lavages and ascitic liquid from 25 CRC patients and 25 control patients, respectively. We could confirm that the isolation and enrichment of EVs from peritoneal lavages and ascitic fluid was successful, obtaining a major population of vesicles with a mean mode of 163.9 (measured by Nanosight®). Our study shows that EV-associated miRNAs can be consistently extracted from peritoneal lavages and ascitic liquids and that miRNA expression profiles can indicate and represent the status of CRC patients. Taqman OpenArray technology permit us to analyze the EV-associated miRNA and the differential expression analysis yielded a list of 210 miRNAs that were significantly dysregulated in CRC patients, being downregulated the 98.57% of the altered miRNAs (only 3 miRNA were upregulated whilst the rest of miRNAs were downregulated in cancer patients). In CRC, most of the previous studies of miRNAs dysregulated were mostly performed in tissue specimens [123] but also in different body fluids as plasma or serum. The first report detected 69 miRNAs in serum of CRC patients but not in serum of controls [124]. Since then, several studies have identified miRNA upregulation or downregulation in plasma or serum samples [123] including studies that have focus on the search of biomarkers in miRNAs dysregulated in the vesicular fraction of the serum or plasma of CRC patients. Hiroko Ogata-Kawata et al [125] analyzed the EV-associated miRNA

profile of serum samples from CRC patients and healthy controls and identified 16 miRNA that were expressed in a significantly higher levels among CRC patients. Of these, 7 miRNAs were suggested as promising diagnostic biomarkers of CRC with an AUC between 0.67 and 0.95. More recently, the serum exosomal miRNA-19a was found to be upregulated in the serum of CRC patients compared to healthy volunteers, but also was associated with poor prognosis [126]. However, none of these potential biomarkers have been translated to the clinic. To the best of our knowledge, our study is the first to report the value of this proximal fluid for the identification of miRNAs associated to EVs in CRC, unveiling the promising use of the top-10 miRNA dysregulated, miRNA-199b-5p, miRNA-150-5p, miRNA-29c-5p, miRNA-218-5p, miRNA-99a-3p, miRNA-383-5p, miRNA-199a-3p, miRNA-193a-5p, miRNA-10b-5p and miRNA-181c-5p, as diagnostic biomarkers showing the AUC value higher than 0.95.

Finally, in **Chapter 3- "Identification of EV-associated miRNAs biomarkers for LC in pleural lavage"-** we focused our efforts on the study of the EV-associated miRNA profile of 25 control pleural fluids and 21 pleural lavages from LC patients by using the Taqman OpenArray technology. The isolation and enrichment of EVs from pleural lavages and pleural fluid was successfully confirm by Nanosight® obtaining a major population of vesicles with a mean mode of 186.1. The differential expression analysis between the two groups yielded a list of 14 miRNAs that were significantly dysregulated, among those, 5 miRNAs were upregulated and 9 miRNAs were downregulated. The best diagnostic biomarkers were miRNA-1-3p, miRNA-144-5p and miRNA-150-5p with an accuracy to label diagnose for LC of 0.941, 0.882 and 0.912, respectively. In our study, miR-1-3p was found to be downregulated which is in accordance to observations by other groups that have investigated the role of miR-1-3p as a tumor-suppressed miRNA in different types of cancer [127, 128 129 130]. Specifically, in LC, Nasser et al showed that miR-1 expression is reduced in LC and inhibits the tumorigenic potential of LC cells by down-regulating oncogenic targets

[131]. Also in accordance with our finding, many studies in several types of cancers have reported that miR-144-3p acted as an antitumor miRNA [132, 133] and specifically, in LC has been suggested to play an important role in NSCLC cell radiosensitivity [134]. MiRNA-150-5p, which was upregulated by a 3-fold expression in LC patients, has been found to be upregulated in tissue suggesting that miRNA-150-5p may be involved in the pathogenesis of LC as an oncogene [135, 136, 137]. However, studies in other types of cancer, i.e. glioma [138], cholangiocarcinoma (CCA) [139] and colon cancer [140], showed a tumor suppressor role of miRNA-150-5p. In vitro experiments on regulation of CCA found that miR-150-5p overexpression inhibited tumor cell proliferation, migration, and invasion capacity, whereas knockdown of miR-150-5p expression induced tumor cell proliferation, migration, and invasion [139]. In the clinical setting, our study provides the evidence that the use of EV-associated miRNA isolated from pleural lavages and pleural liquid are a potential source of biomarkers for LC. Importantly, our study unveiled the promising use of miRNA-1-3p, miRNA-144-5p and miRNA-150-5p with an accuracy to label diagnose for LC of 0.941, 0.882 and 0.912, respectively.

The analysis of the profile of peritoneal and pleural lavages from EC, CRC and LC, respectively allowed the generation of three miRNA signatures dysregulated in cancer conditions. Although we found a set of miRNAs highly specific for each cancer type, the majority of them are common in EC, CRC and LC patients. Importantly and thanks to a bioinformatic study that was performed using Gene Ontology using the whole list of significant miRNAs, we could show that most of these dysregulated miRNAs has been related with some biological and molecular functions. The fact that they are shared by EC, CRC and LC and probably related with and important process of cancer opens a new avenue to investigate the possibly relation of these miRNAs in some of the process keys for the development of a cancerous process.

Our studies are conceived as the first step to prove the potential use of the EV-miRNAs isolated from pleural and peritoneal fluids for the detection of EC, CRC and LC biomarkers. Nevertheless, the three studies have common limitations that need to be discussed and tackled in future research studies.

This work permitted to identify a large number of dysregulated miRNAs associated with EVs in the peritoneal lavage obtained from EC and CRC patients and miRNAs associated with EVs in the pleural lavage of LC patients. Nevertheless, the sample size that was used is limited, and the false discovery rate of a discovery phase study is then very high. Thus, these promising biomarkers should be further validated as well as combined in order to increase the already excellent accuracy of each individual miRNAs. This should be done in an independent study including a larger cohort of patients and controls. Importantly, the control patients group of the three studies was limited by the fact that it is not ethically and technically possible to perform pleural and peritoneal lavages by surgery in normal patients without any pathologic conditions and that is the main reason why we had to select patients with some non-cancerous pathology. A broad spectrum of non-cancer patients should be considered in future studies.

The design of the study based on EV-associated miRNAs from peritoneal lavage of EC patients vs control patients could have been refined. The control patients group was limited by the fact that we included a large proportion of male patients, whilst the cancer group was composed only by women. As this might be an important limiting factor, we already evaluated if the gender factor was affecting our results and we found out that this was not a relevant factor for most of our significant miRNAs (see Figure 16). On the one hand, to rule out the gender bias, we identified miRNAs that were significantly deregulated in the endometrial cancer (EC) patients vs. only the 4 control females. There were 8 such miRNAs (adj. p-value < 0.05 and abs(logFC) >= 1): miRNA-383-5p, miRNA-10b-5p, miRNA-34c-3p, miRNA-449b-5p, miRNA-34c-

5p, miRNA-200b-3p, miRNA-2110 and miRNA-34b-3p. All of them were also down-regulated in cancer vs. full control (men + women) and among which there were three top performers - miRNA-383-5p, miRNA-34c-3p, miRNA-34c-5p – discriminating cancer patients against all control subjects. On the other hand, we generated boxplots with the expression dCT of the most significant miRNA dividing our control dataset according to gender. This permitted us to visualize if the pattern of expression in males differ from the ones observed in females.

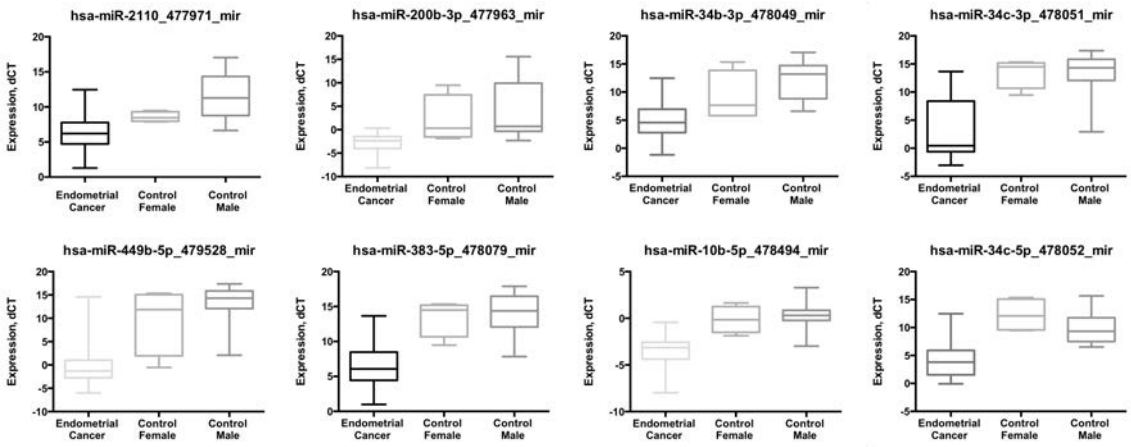

Figure 16. Diagnostic performance of the top-8 differentially expressed miRNAs. Relative dCT values of top differentially expressed miRNAs in patients with EC (n=22) compared to control female patients (n=4) and control male patients (n=18).

In Figure 17, the bar plots of the top significant miRNAs are shown to demonstrate that the expression of those miRNAs was not affected by the gender factor. This approache allowed us to be confident that the gender factor was not a relevant factor in our results and to proceed with the current heterogeneous control group for the classification analysis, which otherwise would be impossible with a small female-only control group. Although we tested that differential miRNAs were not dependent on the gender factor, further studies should include only female controls.

The future validation should be focused on the use of this peritoneal and pleural fluid as an untapped source of biomarkers rather than to validate them in tissue specimens. To do that, the use of Nanostring technology or other targeted high-throughput technologies that can be applied in miRNAs extracted from body fluids might be optim.

In summary, **the work enclosed in this thesis permitted us to unveil that the peritoneal and pleural fluids are an excellent source of biomarkers and can provide insights about the alterations found in EC, CRC and LC. We strongly believe that this study is relevant to the scientific community since the use of this proximal fluids were not previously reported, and might open new avenues.**

CONCLUSIONS

The main conclusions derived from this thesis are:

1.- Extracellular vesicles exist in the peritoneal and pleural lavage as in ascitic and pleural fluid and can be isolated by a protocol based on differential ultracentrifugation.

2.- The enriched cargo of miRNAs contained in extracellular vesicles derived from peritoneal and pleural fluids offers new opportunities for the discovery of miRNA biomarkers of endometrial, colorectal and lung cancers.

3.- The OpenArray technology allows to investigate up to 754 miRNAs. In our study, we were able to detect up to 49.2%, 49.2% and 38.2% of those miRNAs in extracellular vesicles from peritoneal and pleural lavages of endometrial, colorectal and lung cancer patients, respectively.

4.- The expression analysis of EV-miRNAs isolated from peritoneal lavages yielded 114 dysregulated miRNAs between control and endometrial cancer cases. Among those, 96 miRNAs were downregulated and 18 miRNAs were upregulated in peritoneal lavages from endometrial cancer patients comparted to non-cancer patients.

5.- MiRNA-383-5p, miRNA-10b-5p, miRNA-34c-3p, miRNA-449b-5p, miRNA-34c-5p, miRNA-200b-3p, miRNA-2110 and miRNA-34b-3p. All of them demonstrated a great potential as diagnostic biomarkers as they had an AUC value higher than 0.90.

6.- The expression analysis of EV-miRNAs isolated from peritoneal lavages yielded 210 dysregulated miRNAs between control and colorectal cancer cases. Only 3 miRNAs were upregulated, whilst the rest was downregulated in colorectal patients.

7.- The top-10 dysregulated miRNAs in peritoneal lavages from colorectal cancer patients were miRNA-199b-5p, miRNA-150-5p, miRNA-29c-5p, miRNA-218-5p, miRNA-99a-3p, miRNA-383-5p, miRNA-199a-3p, miRNA-193a-5p, miRNA-10b-5p and miRNA-181c-5p. All of them demonstrated a great potential as diagnostic biomarkers as they had an AUC value higher than 0.95.

8.- The expression analysis of EV-miRNAs isolated from pleural lavages yielded 14 dysregulated miRNAs between control and lung cancer cases. Among those, 5 miRNAs were upregulated and 9 miRNAs were downregulated.

9.- MiRNA-1-3p, miRNA-144-5p and miRNA-150-5p presented the highest accuracy to detect lung cancer, 0.941, 0.882 and 0.912, respectively in pleural lavages.

10.- In conclusion, as a whole this thesis encompasses a wide spectrum of translational research that was useful to prove the potential of miRNAs isolated from EVs on proximal bodily fluids, specifically on peritoneal fluid to improve the clinical management of the endometrial and colorectal cancer patients, and in pleural fluid to improve the clinical management of lung cancer patients.

JOURNAL OF PUBLICATIONS

This thesis resulted in the publication of six manuscripts. Three of them (D, E and F) are included as part of the thesis, and another 3 articles were published thanks to my collaboration in other research projects:

Publication A) Piulats JM, Guerra E, Gil-Martín M, **Roman-Canal B**, Gatius S, Sanz-Pamplona R, Velasco A, Vidal A, Matias-Guiu X. "Molecular approaches for classifying endometrial carcinoma", Gynecol Oncol. 2017 Apr;145(1):200-207. doi: 10.1016/j.ygyno.2016.12.015. Epub 2016 Dec 29. Review.

Publication B) Gatius S, Cuevas D, Fernández C, **Roman-Canal B**, Adamoli V, Piulats JM, Eritja N, Martin-Satue M, Moreno-Bueno G, Matias-Guiu X. "Tumor Heterogeneity in Endometrial Carcinoma: Practical Consequences", Pathobiology. 2018;85(1-2):35-40. doi: 10.1159/000475529. Epub 2017 Jun 15.

Publication C) Cuevas D, Valls J, Gatius S, **Roman-Canal B**, Estaran E, Dorca E, Santacana M, Vaquero M, Eritja N, Velasco A, Matias-Guiu X. "Targeted sequencing with a customized panel to assess histological typing in endometrial carcinoma", Virchows Arch. 2019 May;474(5):585-598. doi: 10.1007/s00428-018-02516-2. Epub 2019 Feb 1.

Publication D) Roman-Canal B, Moiola CP, Gatius S, Bonnin S, Ruiz-Miró M, González E, González-Tallada X, Llordella I, Hernández I, Porcel JM, Gil-Moreno A, Falcón-Pérez JM, Ponomarenko J, Matias-Guiu X, Colas E. "EV-Associated miRNAs from Peritoneal Lavage are a Source of Biomarkers in Endometrial Cancer", Cancers (Basel). 2019 Jun 18;11(6). pii: E839. doi: 10.3390/cancers11060839.

Publication E) Roman-Canal B, Tarragona J, Moiola CP, Gatius S, Bonnin S, Ruiz-Miró M, Sierra JE, Rufas M, González E, Porcel JM, Gil-Moreno A, Falcón-Pérez JM, Ponomarenko J, Matias-Guiu X, Colas E. "EV-associated miRNAs from peritoneal lavage as potential diagnostic biomarkers in colorectal cancer", J Transl Med. 2019 Jun 20;17(1):208. doi: 10.1186/s12967-019-1954-8.

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