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Review Article

Extracellular MicroRNA in liquid biopsy: applicability in cancer diagnosis and prevention

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Abstract: One of the goals of contemporary cancer research is the development of new markers that facilitate earlier and non-invasive diagnosis. MicroRNAs are non-coding RNA molecules that regulate gene expression; studies have shown that their expression levels are altered in cancer. Recently, extra-cellular microRNAs have been detected in biological fluids and studied as possible cancer markers that can be detected by noninvasive procedures. In this review, we analyze the current understanding of extracellular miRNAs based on clinical studies to establish their possible use for the prevention of the most common tumors. Despite discrepancies among different studies of the same cancers, panels of specific extracellular microRNAs are emerging as a new tool for the secondary (selection of high-risk individuals to undergo screening) and tertiary (relapse) prevention of cancer.

Keywords: Biomarker, cancer, diagnosis, miRNA, therapy, outcome, prognosis, exsosome, microvescicle, prevention

Introduction

Cancer diagnosis is based on radiology, which exposes the patient to radiation danger, and biopsy, an invasive process that exposes the patient to complications such as hemorrhages [1, 2].

Serological cancer biomarkers, such as CEA, CA 125 and CA 19.9, are commonly employed in follow up but have low specificity and sensitivity as alternatives to imaging and biopsy [3, 4].

Accordingly, new low-cost and non-invasive cancer biomarkers need to be developed to improve screening protocols and therapy and to provide information on chemoresistance and the risk of relapses. Therefore, one of the goals of cancer research is the identification of new biomarkers to provide less invasive, less dangerous and less expensive but more informative screening protocols. For example, ongoing strategies for breast cancer screening reportedly do not reduce mortality but do increase overdiagnosis, i.e., the detection of cancers that will not cause death [5].

Similar considerations have long been established for prostate cancer [6]. Liquid biopsy is a novel concept that relies on the study of circulating cells or cell components in the blood to obtain a diagnosis without the need for invasive techniques [7]. A liquid biopsy uses body fluids as surrogate tissues to provide information on cancer target tissues.

The term *liquid biopsy* was originally used to represent the study of circulating cells, but the term has since been extended to other cell components, such as circulating DNA, microRNA (miRNA), microvesicles, and exosomes [7].

As described in **Figure 1**, a liquid biopsy, like a classical biopsy, can be employed for early diagnosis, tumor staging, estimating the risk of metastasis and relapses, and monitoring therapy [7].

miRNAs are single-stranded, non-coding RNA molecules that are 20-23 nucleotides long and that act as master regulators of gene-expression at the post-transcriptional level [8]. miRNAs were initially described in *C. elegans* by Lee in 1993 [9].

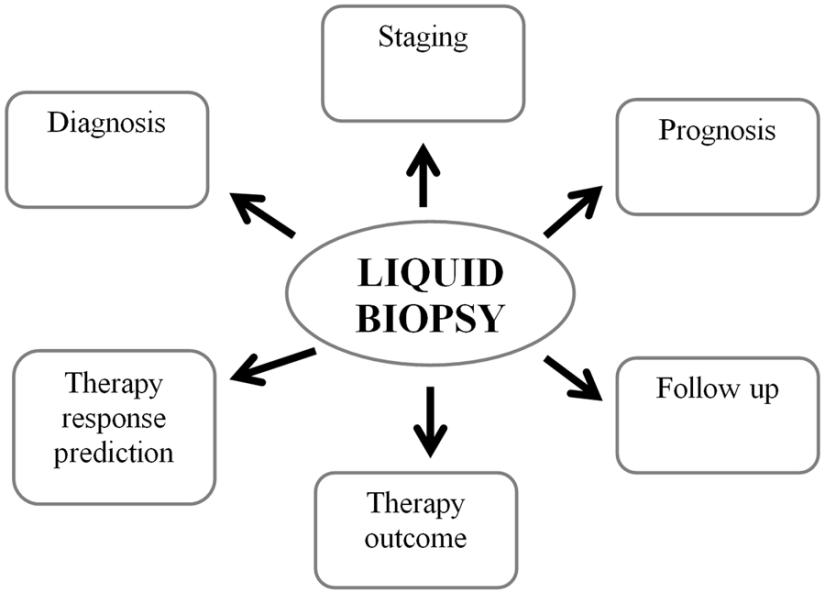

Figure 1. Liquid biopsy is the analysis in biological fluids of circulating cells or other cellular components, such as miRNAs, to provide information on cancer appearance and development in target tissues. The liquid biopsy in oncology may be used for early diagnosis (secondary prevention), cancer staging, prognosis, predicting the response to a specific therapy, evaluating therapy outcome, and patient follow up to individuate early relapses (tertiary prevention).

The human genome contains more than 2,500 mature miRNAs [10]. A single miRNA influences the expression of several genes, although a single gene may be influenced by several miRNAs. This situation generates a complex network, and the analysis of miRNA panels is consequently more efficient in cancer studies than the analysis of a single miRNA [11, 12].

miRNAs are initially expressed as precursors (pre-miRNAs) in the form of dsRNA hairpins and are then cleaved in the nucleus by the ribonuclease *Drosha* to produce primary miRNAs (pri-miRNAs). Pri-miRNAs are transported by Exportin-5 from the nucleus to the cytoplasm, and the terminal loop is then cleaved by Dicer RNase III to form a double-stranded mature RNA. Dicer initiates the formation of the RNA-induced silencing complex (RISC), which is responsible for gene silencing [13].

miRNAs are distributed throughout the human genome, but the majority are located at fragile sites that are frequently deleted in human cancers [14]. Therefore, irreversible miRNA alterations drive cancer development and progression [15].

The clustering of miRNA expression profiles for similar tumor types is more accurate than that of messenger RNA expression [16] because less than 5% of messenger RNA is translated into proteins, whereas 100% of miRNAs are biologically relevant [17].

miRNAs were originally studied in tissues, but they have also been observed in the blood, urine and other body fluids [18]. This finding was initially unexpected because blood and other fluids contain RNAses, specific enzymes that cleave RNA and consequently exert an antiviral function in these liquids [18]. However, the mechanisms by which miRNA evades RNAse have remained elusive.

miRNAs can escape RNAse activity in two ways. Some miRNAs circulate in the blood and other biological fluids in exosomes, membrane vesicles created by cells to expel specific material by fusing multivesicular bodies with the plasma membrane [19]. Exosomes are generally small (30-200 nm) and participate in inter-cellular communication. Oligonucleotides are protected from RNAses in exosomes because these enzymes cannot penetrate the vesicles. In microvesicles (250-400 nm), oligonucleotides are enveloped by a plasma membrane that includes cellular receptors.

Most miRNAs were recently shown to circulate in the blood in a free form associated with a ribonucleoprotein complex or argonaute-2, which protects them from RNAse activity [20, 21].

Circulating miRNAs were first hypothesized to derive from necrotic cells and blood cells, but evidence suggests that miRNAs are secreted by cells to communicate and influence gene expression in nearby or distant tissues [22, 23].

miRNAs are present in biological fluids in both microvesicles and free oligonucleotides, but

Table 1. Extracellular miRNAs associated with colorectal cancer. miRNAs forcolorectal cancer were isolated in blood and were used as diagnostic andprognostic biomarkers

miRNA	Liquid	Expression	Use	Number of examined subjects	Reference
miR-17-3p	Plasma	Overexpression	Diagnosis	45	[16]
miR-17-3p	Serum	Overespression	Diagnosis; Prognosis	40	[42]
miR-17-92a	Serum	Overexpression	Therapeutic Outcome	299	[165]
miR-18a	Plasma	Overexpression	Early Diagnosis	324	[38]
miR-19a	Serum	Overexpression	Therapeutic Outcome	72	[44]
miR-19a	Serum	Overexpression	Therapeutic Outcome	299	[165]
miR-19a-3p	Serum	Overexpression	Early Diagnosis	697	[166]
miR-20a	Plasma	Overexpression	Early Diagnosis	324	[38]
miR-21	Plasma	Overexpression	Early Diagnosis	98	[33]
miR-21	Serum	Overexpression	Diagnosis; Prognosis; Therapeutic Outcome	186	[34]
miR-21	Serum	Overexpression	Diagnosis; Prognosis; Therapeutic Outcome	213	[167]
miR-21	Serum	Overexpression	Diagnosis; Prognosis; Therapeutic Outcome	237	[36]
miR-21	Serum	Overexpression	Diagnosis; Prognosis	224	[35]
miR-21a	Plasma	Overexpression	Early Diagnosis	324	[38]
miR-23a	Serum	Overexpression	Early Diagnosis	102	[32]
miR-24	Plasma	Underexpression	Diagnosis; Therapeutic Outcome	353	[37]
miR-27b	Plasma	Overexpression	Therapeutic Outcome	24	[41]
miR-29a	Plasma	Overexpression	Early Diagnosis	324	[38]
miR-29a	Serum	Overexpression	Early Diagnosis	237	[36]
miR-29b	Serum	Underexpression	Diagnosis	110	[168]
miR-30b	Serum	Underexpression	Prognosis	41	[39]
miR-30c	Serum	Underexpression	Prognosis	41	[39]
miR-30d	Serum	Underexpression	Prognosis	41	[39]
miR-92	Plasma	Overexpression	Diagnosis	45	[16]
miR-92a	Plasma	Overexpression	Early Diagnosis	324	[38]
miR-92a-3p	Serum	Overexpression	Early Diagnosis	697	[166]
miR-106a	Plasma	Overexpression	Therapeutic Outcome	24	[41]
miR-106a	Serum	Overesxpression	Diagnosis; Prognosis	40	[42]
miR-106b	Plasma	Overexpression	Early Diagnosis	324	[38]
miR-125b	Serum	Overexpression	Early Diagnosis	237	[36]
miR-126	Plasma	Overexpression	Therapeutic Outcome	68	[43]
miR-126	Serum	Underexpression	Diagnosis; Prognosis	224	[35]
miR-130b	Plasma	Overexpression	Therapeutic Outcome	24	[41]
miR-133a	Plasma	Overexpression	Early Diagnosis	324	[38]
miR-141	Serum	Overexpression	Diagnosis; Prognosis	224	[35]
miR-143	Plasma	Overexpression	Early Diagnosis	324	[38]
miR-145	Plasma	Overexpression	Early Diagnosis	324	[38]
miR-145	Serum	Overexpression	Early Diagnosis	60	[169]
miR-146a	Serum	Underexpression	Prognosis	41	[39]
miR-148a	Plasma	Overexpression	Therapeutic Outcome	24	[41]
miR-155	Serum	Overexpression	Diagnosis; Prognosis	206	[170]
miR-181b	Plasma	Overexpression	Early Diagnosis	324	[38]
miR-183	Plasma	Overexpression	Diagnosis	179	[171]
miR-193a-3p	Serum	Overexpression	Early Diagnosis	102	[32]
miR-194	Serum	Underexpression	Diagnosis	110	[168]
miR-223-3p	Serum	Overexpression	Early Diagnosis	697	[166]

miR-320a	Plasma	Underexpression	Diagnosis; Therapy Outcome	353	[37]
miR-326	Plasma	Overexpression	Therapeutic Outcome	24	[41]
miR-338-5p	Serum	Overexpression	Early Diagnosis	102	[32]
miR-342-3p	Plasma	Overexpression	Early Diagnosis	324	[37]
miR-372	Serum	Overexpression	Early Diagnosis	NA	[172]
miR-422a	Serum	Overexpression	Early Diagnosis	697	[166]
miR-423-5p	Plasma	Underexpression	Diagnosis; Therapy Outcome	353	[37]
miR-484	Plasma	Overexpression	Therapeutic Outcome	24	[41]
miR-532-3p	Plasma	Overexpression	Early Diagnosis	324	[38]
miR-885-5p	Serum	Overexpression	Prognosis	169	[40]

the form depends on the nature of the cell that releases the miRNA: secreting cells release miRNAs in microvesicles, whereas other cell types release miRNAs as free oligonucleotides without an exosome [20].

miRNAs are ubiquitous throughout all biological fluids, including cerebrospinal fluid (CSF), urine, blood and breast milk. The miRNA concentration in these fluids may be altered by physiological and pathological processes, such as pregnancy or tumors. It was demonstrated that also tumor cells have also been shown to release miRNA [19] according to specific miRNA patterns. Tumors alter the normal concentrations of miRNAs in biological fluid. Thus, these oligonucleotides may serve as cancer biomarkers [18].

In this review we analyze studies of miRNAs obtained via liquid biopsy for the most common tumors. Specifically, we detail the miRNAs that have been isolated and their associated uses.

Colorectal cancer

Colorectal cancer is the third most frequent tumor worldwide [24] and represents the second most common cause of death by cancer in Western countries [25]. Worldwide, colorectal cancer has been estimated to cause more than 700,000 deaths annually [26, 27].

Early diagnosis is based on the fecal occult blood test, a relatively low-cost screening test with poor sensitivity and specificity if dietary restriction is not pursued [28]. Accordingly, the gold standard for the early diagnosis of colon cancer is endoscopic biopsy, an invasive procedure reserved for high-risk individuals.

The use of other diagnostic imaging tests, such as computed tomography (CT), is limited by radiation exposure and high costs [29]. Serum markers, such as CEA and CA15.5, are primarily used during follow-up because of their poor specificity [30].

Surgical resection is the primary therapeutic approach and is generally associated with adjuvant and neo-adjuvant chemotherapy or radiotherapy, but 20-30% of patients develop recurrent disease with poor prognoses [31]. Early detection and surgical approaches ensure good prognoses [32], and early diagnosis, insofar mainly based on the fecal occult test, is consequently fundamental to reduce death due to this malignancy.

Extracellular miRNA associated with colorectal cancer has been studied mainly in circulating blood, plasma and serum (**Table 1**); most of these studies have analyzed blood serum. A recent meta-analysis demonstrated that the serum-based miRNA assay is more accurate than a plasma-based assay in colon adenocarcinoma [11].

In an effort to reduce mortality, several miRNAs related to colorectal cancer have been analyzed to identify new markers for early diagnosis (view **Table 1**). The most important of these miRNAs is miR-21, whose overexpression in the serum and plasma [33] is associated with malignancy and directly correlates with tumor size [34-36]. miR-21 is also an independent prognostic factor for colorectal cancer [34].

miR-24, miR-320a and miR-423-5p are interesting diagnostic markers because their expression levels are decreased in the sera of colorectal cancer patients but increased the sera of inflammatory bowel disease patients. Therefore, these miRNAs are reliable non-invasive markers for differential diagnosis [37]. Other miRNAs, such as miR-106a, miR-106b, miR-29a, have been studied for the early diag-

Table 2. Panels of extracellular miRNAs used as biomarker in colorectal cancer. Multiples miRNAs have been analyzed together to increase their sensitivity and the specificity as biomarker. miRNAs may be associated also with other biomarkers such as CEA

MiRNA panel	Use	Fluid	Reference
miR-17-3p; miR-106a	Diagnosis; Prognosis; Therapy Outcome	Serum	[42]
miR-18a; miR-20a; miR-21; miR-29a; miR-92a; miR-106b; miR-133a; miR-143; miR-145; miR-181b; miR-342-3p; miR-532-3p	Early Diagnosis	Plasma	[38]
miR-193a-3p; miR-23a; miR-338-5p	Early Diagnosis	Serum	[32]
miR-19a; CEA	Therapeutic Outcome	Serum	[44]
miR-19a-3p; miR-223-3p; miR-92a-3p; miR-422a	Early Diagnosis	Serum	[166]
miR-21, miR-29a, miR-125b	Diagnosis; Prognosis; Therapy Outcome	Serum	[36]
miR-126; miR-141; miR-21	Dignosis; Liver Metastasis Detection	Serum	[35]

nosis colorectal cancer, but, as evidenced by a meta-analysis by Wang, single miRNAs exhibit low specificity or sensitivity, which necessitates the use of miRNA panels [11].

Table 2 shows the most important miRNA panel associations proposed for colon cancer. Some authors combined miRNAs that were differentially expressed in their studies, such as in Yamada's model [36], whereas other authors use a combination of 10-12 miRNAs identified based on the available literature on colon cancer, such as in Luo's panel [38].

Generally, the miRNA concentration poorly correlates with TNM stage [38]. However, miR-193a-3p, miR-23a and miR-338-5p have been found to relate to the TNM stage [32], which ensures their relevance as diagnostic and prognostic markers.

miRNAs have also been studied as prognostic markers. For example, miR-126 and miR141 have been related to liver metastasis and proposed for the early detection of these metastases [35]. Conversely, miR-146a, miR-30b, miR-30c, miR-885-5p and miR-30d are downregulated in advanced colon adenocarcinomas, which indicates a poor prognosis [39, 40].

miRNAs have also been evaluated as predictors of therapy outcome. Kjersem identified 6 miRNAs associated with chemotherapy outcome. Increases in miR-326 have been associated with shortened overall survival and progression-free survival after 5-fluoruracil- and oxaliplatin-based chemotherapy [41]. Increases in miR-17-3p and miR-106a levels in the blood serum have been correlated with poor prognosis and poor therapeutic outcome [42]. miR-126 was studied in patients treated with bevacizumab, and its overexpression in the blood during treatment was associated with a poor response [43].

miR-19a upregulation has been employed to distinguish chemotherapy-resistant from non-chemoresistant patients and was complementary to CEA, which suggests that these two markers could be analyzed together to increase the testing sensitivity and specificity [44].

Lung cancer

Lung cancer is the most common cause of cancer death worldwide [45]. It has a 5-year survival rate of less than 15%, and the most important clinical determinants of lung cancer are its staging and histopathological classification [46].

Approximately 80% of patients have non-small-cell lung cancer (NSCLC), the incidence of which is increasing [45]. The most common subtypes are adenocarcinoma and squamous cell carcinoma [47].

Thirteen percent of lung cancer cases are small-cell lung cancer, a tumor with a very poor prognosis. The high mortality related to small-cell lung cancer is due to its chemoresistance, relapse frequency and high metastatic potential [48].

Lung cancer is generally treated with surgical resection, but a sub-population of patients benefits from adjuvant chemotherapy. Patients with clinical stages Ib to IIIa may receive both surgery and adjuvant chemotherapy [46]. Despite diagnostic and therapeutic improvements, the prognosis of lung cancer remains poor, especially because the disease is usually diagnosed in its advanced stages, when metastases are present [45].

Table 3. Extracellular miRNAs associated with lung cancer. miRNAs for lung cancer were isolated in blood, BAL, pleural effusion, and sputum and were used as diagnostic and prognostic biomarkers

miRNA	Liquid	Expression	Use	Number of examined subjects	Reference
let-7a	Serum	Underexpression	Early Diagnosis	164	[54]
let-7b	Serum	Underexpression	Early Diagnosis	164	[54]
let-7d	Serum	Underexpression	Early Diagnosis	164	[54]
let-7f	Plasma	Underexpression	Diagnosis; Staging; Prognosis	48	[173]
miR-1	Serum	Underexpression	Diagnosis	303	[46]
miR-10b	Serum	Overexpression	Prognosis	361	[55]
miR-17	Serum	Underexpression	Early Diagnosis	164	[54]
miR-20b	Plasma	Underexpression	Diagnosis	48	[173]
miR-21	Sputum	Na	Diagnosis	31	[174]
miR-21	BAL	Na	Diagnosis	31	[174]
miR-21	Serum	Overexpression	Prognosis	361	[55]
miR-22	Serum	Underexpression	Early Diagnosis	164	[54]
miR-22	Blood	Overexpressed	Diagnosis; Prognosis; Therapy outcome	49	[175]
miR-22	Pleural effusion	Underexpressed	Diagnosis	87	[176]
miR-24	Blood	Overexpressed	Diagnosis	49	[175]
miR-26a	Serum	Underexpression	Early Diagnosis	164	[54]
miR-26b	Serum	Underexpression	Early Diagnosis	164	[54]
miR-28-5p	Serum	Underexpression	Early Diagnosis	164	[54]
miR-29a	Serum	Overexpression	Early Diagnosis	164	[54]
miR-29c	Serum	Overexpression	Diagnosis	118	[52]
miR-30b	Serum	Underexpression	Early Diagnosis	164	[54]
miR-30c	Serum	Underexpression	Early Diagnosis	164	[54]
miR-30c	Serum	Underexpression	Early Diagnosis	1035	[177]
miR-30d	Serum	Overexpression	Diagnosis	303	[46]
miR-30e-3p	Plasma	Underexpression	Diagnosis; Staging; Prognosis	48	[173]
miR-32	Serum	Overexpression	Early Diagnosis	164	[54]
miR-34	Blood	Overexpressed	Diagnosis	49	[175]
miR-92a	Plasma	Underexpression	Early Diagnosis	1035	[177]
miR-92a	Serum	Underexpression	Early Diagnosis	164	[54]
miR-98	Serum	Underexpression	Early Diagnosis	164	[54]
miR-103	Serum	Underexpression	Early Diagnosis	164	[54]
miR-126	Serum	Underexpression	Early Diagnosis	164	[54]
miR-133b	Serum	Overexpression	Early Diagnosis	164	[54]
miR-134	Pleural Effusion	Underexpressed	Diagnosis	87	[176]
miR-139-5p	Serum	Underexpression	Early Diagnosis	164	[54]
miR-140-5p	Plasma	Overexpression	Early Diagnosis	1035	[177]
miR-140-5p	Serum	Overexpression	Early Diagnosis	164	[53
miR-141	Serum	Overexpression	Early Diagnosis	154	[47]
miR-141	BAL	Overexpressed	Diagnosis	91	[178]
miR-142-3p	Serum	Underexpression	Early Diagnosis	164	[54]
miR-142-5p	Serum	Overexpression	Early Diagnosis	164	[54]
miR-143	Sputum	Na	Diagnosis	31	[174]
miR-143	BAL	Na	Diagnosis	31	[174]
miR-148a	Serum	Overexpression	Early Diagnosis	164	[54]
miR-148b	Serum	Underexpression	Early Diagnosis	164	[54]
miR-155	Sputum	Na	Diagnosis	31	[174]

miR-155	BAL	Na	Diagnosis	31	[174]
miR-185	Pleural Effusion	Underexpressed	Diagnosis	87	[176]
miR-191	Serum	Underexpression	Early Diagnosis	164	[54]
miR-193b	Serum	Overexpression	Early Diagnosis	154	[47]
miR-200b	Serum	Overexpression	Early Diagnosis	154	[47]
miR-204	Plasma	Underexpression	Prognosis	176	[179]
miR-210	Sputum	Na	Diagnosis	31	[174]
miR-210	BAL	Na	Diagnosis	31	[174]
miR-223	Serum	Overexpression	Early Diagnosis	164	[54]
miR-301	Serum	Overexpression	Early Diagnosis	154	[47]
miR-328	Serum	Underexpression	Early Diagnosis	164	[54]
miR-331-3p	Serum	Underexpression	Early Diagnosis	164	[54]
miR-342-3p	Serum	Underexpression	Early Diagnosis	164	[54]
miR-372	Sputum	Na	Diagnosis	31	[174]
miR-372	BAL	Na	Diagnosis	31	[174]
miR-374a	Serum	Underexpression	Early Diagnosis	164	[54]
miR-376a	Serum	Underexpression	Early Diagnosis	164	[54]
miR-429	Serum	Underexpression	Diagnosis; Prognosis	118	[52]
miR-432	Serum	Overexpression	Early Diagnosis	164	[54]
miR-484	Serum	Underexpression	Early Diagnosis	164	[54]
miR-486	Serum	Overxepression	Diagnosis	303	[46]
miR-486-5p	Plasma	Underexpression	Early Diagnosis	1035	[177]
miR-486-5p	Plasma	Underexpression	Prognosis	1035	[177]
miR-486-5p	Serum	Underexpression	Early Diagnosis	164	[54]
miR-499	Serum	Underexpression	Diagnosis	303	[46]
miR-566	Serum	Overexpression	Early Diagnosis	164	[54]
miR-652	Serum	Overexpression	Diagnosis	366	[53]
miR-660	Serum	Overexpression	Diagnosis	1035	[177]
miR-660	Serum	Overexpression	Diagnosis	366	[53]

Diagnosis is based on biopsy and radiography, but radiography and CT cannot ensure an early diagnosis [45]. Furthermore, low-dose CT also cannot provide an early diagnosis because it has a high incidence of false positives and is expensive [47]. To employ CT for the secondary prevention of lung cancer, we need to increase its specificity and to reduce the number of subjects to limit its cost. For example, CT may be employed to study only patients who smoke. Pastorino et al. studied low-dose spiral CT from 2000 to 2005 in Milan to analyze mortality in smokers. Their evidence showed that annual or biannual screening with low-dose spiral CT does not influence mortality in smokers [49].

The need for follow-up in select high-risk patients underline the interest of serological biomarkers [49]. Like CEA, these markers feature low specificity and sensitivity; therefore, they are only used during follow-up to monitor for relapse (tertiary prevention). Another possible diagnostic approach is bronchoscopy, but its interpretation is complex and affected by low sensitivity [50]. Therefore, this technique is not used to diagnose lung cancer [45, 51].

Furthermore, identifying subgroups of patients more accurately may refine the prognostic model and lead to more personalized cancer treatment [46].

As highlighted in **Table 3**, the serum concentrations of several miRNAs have been associated with lung cancer, especially NSCLC. Most of these oligonucleotides have been studied as early diagnostic markers in an effort to identify novel non-invasive biomarkers.

Measuring the serum concentrations of miRNAs can distinguish healthy patients from lung cancer patients [45], but the accuracy of this

Table 4. Panels of extracellular miRNAs used as biomarker in lung cancer. Multiples miRNAs were analyzed together to increase their sensitivity and the specificity as biomarker. miRNAs may be associated also with other biomarkers such as CEA and Cyfra 21-1

MiRNA panel	Use	Fluid	Reference
miR-29c; miR-429; CEA	Diagnosis	Serum	[52]
let-7a; let-7b; let-7d; miR-103; miR-126; miR-133b; miR-139-5p; miR-140-5p; miR-142-3p; miR-142-5p; miR-148a; miR-148b; miR-17 ; miR-191; miR-22; miR-223; miR-26a; miR-26b; miR-28-5p; miR-29a; miR-30b; miR-30c; miR-32; miR-328; miR-331-3p; miR-342-3p; miR-374a; miR-376a; miR-432; miR-484; miR-486-5p;miR-566; miR-92a; miR-98	Early Diagnosis	Serum	[54]
miR-193b; miR-301; miR-141; miR-200b	Early Diagnosis	Serum	[47]
miR-1; miR-20d; miR-486; miR-499	Diagnosis	Serum	[46]
miR-652; miR-660; Cyfra 21-1	Diagnosis	Serum	[53]

diagnosis is generally not sufficient for use in screening. To increase the accuracy of using miRNAs for diagnosis, they have been combined in panels [46, 47] along with other lung cancer markers, such as CEA [52] and Cyfra 21-1 [53]. These approaches have yielded interesting findings, which suggest that miRNAs can be used for early lung cancer diagnosis (view **Table 4**).

A model of 34 miRNAs was used to follow up high-risk lung cancer patients and could discriminate early-stage NSCLC patients from healthy patients [54].

Recent studies have suggested that miRNAs may also be employed as prognostic markers. For example, miR-429 overexpression in the serum has been associated with poor overall survival in NSCLC patients [52].

miR-10b and miR-21 have been associated with changes in epithelial growth factor receptor expression in cancer cells. Changes in the expression of this membrane receptor suggest a poor prognosis and a poor response to adjuvant therapy with gefitinib, a tyrosine-kinase inhibitor that targets this receptor [55].

The primary problem in using miRNAs as biomarkers for the early diagnosis of lung cancer is their susceptibility to cigarette smoke exposure. Specifically, cigarette smoke influences miRNA expression in tissues, as demonstrated in murine models [56] and humans [57]. Because the vast majority of lung cancer cases are related to cigarette smoke and because cigarette smoke influences miRNA expression, miRNAs are poorly specific biomarkers of lung cancer in smokers.

To solve this problem, experimental evidence indicates that miRNA expression should be considered a dynamic variable. Because cigarette smoke affects miRNA expression profiles, they should be analyzed at multiple instances to observe changes [58].

Cigarette smoke damages DICER and silences different miRNAs, which irreversibly changes the cell phenotype [58]. Nevertheless, these changes are reversible upon smoking cessation at early time points, but not in all subjects [58]. miRNA reversibility may be used to select high-risk patients for screening, such as annual low-dose spiral CT.

Malignant pleural mesothelioma

Malignant mesothelioma is a highly aggressive cancer arising from the mesothelial surfaces of the pleura, pericardium and peritoneum [59].

The most common form is malignant pleural mesothelioma (MPM), which is associated asbestos exposure. The long latency between exposure and clinical diagnosis suggests that the action of asbestos is associated with progressive DNA lesions caused by a chronic inflammation [60, 61].

The three most common MPM histopathologic subtypes are epithelioid, sarcomatoid and biphasic, which are characterized by both epithelioid and sarcomatoid cells [61]. These subtypes are clinically different, but all are characterized by poor prognoses due to the lack of both effective therapies and early prognostic biomarkers, with an overall survival median of twelve months [62].

The diagnosis of mesothelioma is complex and is based on CT imaging [62]. The gold standard is biopsy, which cannot ensure the withdrawal of available and sufficient material [59]. Differential diagnosis may rely on the cytologi-

Table 5. Extracellular miRNAs associated with malignant pleural mesothelioma. miRNAs were isolated in blood and pleural effusion and were used for diagnostic, staging, and prognostic biomarker. The most of studies analyze miRNAs expression, but one study analyzes miRNA methylation

miRNA	Liquid	Expression	Use	Number of examined subjects	Reference
miR-25	Serum	Overexpression	Diagnosis; Staging; Prognosis	103	[61]
miR-26b	Serum	Overexpression	Diagnosis; Staging; Prognosis	103	[61]
miR-29a	Serum	Overexpression	Diagnosis; Staging; Prognosis	103	[61]
miR-34b/c	Serum	Overexpression In Methylated Form	Diagnosis	110	[62]
miR-34b/c	Serum	Methylation	Diagnosis	110	[62]
miR-101	Serum	Overexpression	Diagnosis; Staging; Prognosis	103	[61]
miR-103a-3p	Plasma	Underexpression	Diagnosis; Differential Diagnosis	95	[70]
miR-126	Pleural Effusion	Underexpression	Diagnosis	53	[71]
miR-126	Serum	Underexpression	Diagnosis	94	[60]
miR-126	Serum	Underexpression	Diagnosis	11	[69]
miR-191	Serum	Underexpression	Diagnosis; Staging; Prognosis	103	[61]
miR-21	Pleural Effusion	Overexpression	Diagnosis	53	[71]
miR-223	Serum	Underexpression	Diagnosis; Staging; Prognosis	103	[61]
miR-335	Serum	Overexpression	Diagnosis; Staging; Prognosis	103	[61]
miR-433	Serum	Overexpression	Diagnosis; Staging; Prognosis	103	[61]
miR-516	Serum	Overexpression	Diagnosis; Staging; Prognosis	103	[61]

Table 6. Panels of extracellular miRNAs used as biomarker in malignant pleural mesothelioma. Multiples miRNAs have been analyzed together to increase their sensitivity and the specificity as biomarker. miRNAs may be associated also withother biomarkers such as small mesothelioma related peptides

MiRNA panel	Use	Fluid	Reference
miR-101; miR-25; miR-26b; miR-335; miR-433; miR-191; miR-223; miR-29a; miR-516	Diagnosis; Staging; Prognosis	Serum	[61]
miR-126; small mesothelioma related peptides	Diagnosis	Serum	[60]
miR-103a-3p; mesothelin	Diagnosis; Differential Diagnosis	Plasma	[70]
miR-21; miR-126	Diagnosis	Pleural Effusion	[71]

cal analysis of pleural effusion, but this approach has not received universal acceptance because it cannot distinguish MPM from benign cells, resulting in poor sensitivity [59, 63-65].

MPM radiological diagnosis is also complicated by other histopathological alterations due to asbestos [66]. Few asbestos-exposed subjects develop MPM, which is more commonly associated with other clinical conditions such as pleural plaques. Asbestosis is a disease associated with the inhalation of asbestos and is characterized by pulmonary fibrosis, emphysema and alveolitis with macrophages that exhibit hemosiderin. Asbestos also produces unique fibrotic morphology and pleural effusion, such as retroperitoneal fibrosis and pleural plaques, benign lesions characterized by areas of fibrous thickening on the lining of the lung or near the diaphragm.

These fibrotic lesions complicate and extend the time required for MPM diagnosis, which increases the chance of false positives [66]. Therefore, biomarkers are crucial to facilitate a correct diagnosis without biopsy, which is invasive and carries a risk of death.

However, reliable serological markers are not available for mesothelioma. Several proteins have been proposed as serological biomarkers, such as megakaryocyte potentiating factor, Cyfra21-1, fibulin-3, soluble mesothelin-related peptides and osteopontin, but they have all failed to achieve an acceptable accuracy to be employed in clinical practice [59, 60, 62].

The most interesting of these biomarkers is mesothelin, which is encoded in precursor form and is then post-translationally cleaved into parts: the N terminus is a megakaryocyte

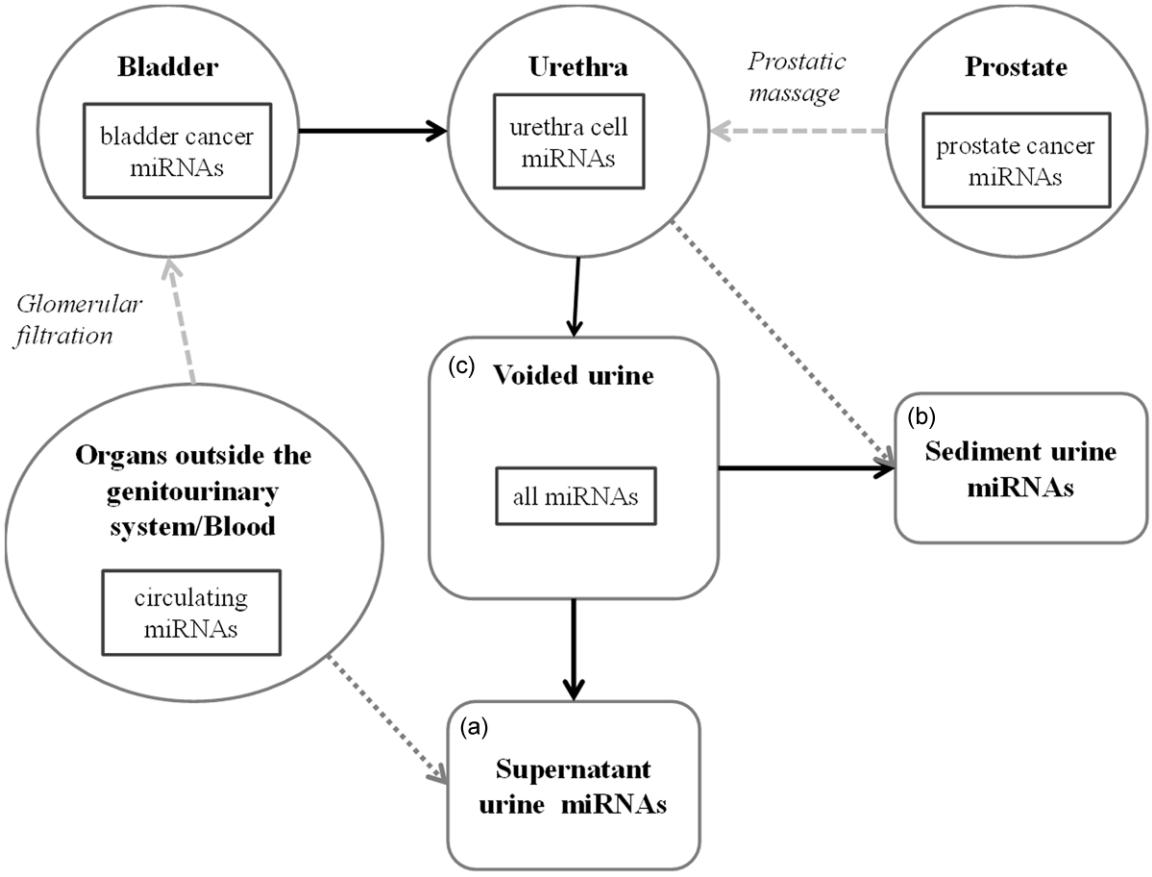

Figure 2. Urine is emerging as a biological fluid suitable to perform liquid biopsy in a minimally invasive manner, a fundamental attribute for secondary and tertiary prevention of cancer. Several extracellular miRNAs have been isolated in urine. It is possible to analyze miRNAs in voided urine, in urine sediment and urine supernatant. The choice of urine fraction is crucial because the miRNAs isolated are quite different: (a) supernatant contains exosomal miRNAs filtered by glomeruli; (b) sediment also contains microRNAs released by different cell types contained in this urine fraction; (c) voided urine contains both miRNA fractions. Because of this reason, supernatant miRNAs have a high accuracy as biomarker of cancer located outside the genitourinary system than sediment miRNAs. In urine it is possible to identify also prostatic miRNAs, especially after a prostate massage. In the circles we indicate the organs where the miRNAs originate from. In squared boxes we indicate the miRNA typologies and in rounded square boxes we indicate urine typologies. Black arrows indicate total miRNA displacement; grey dashed arrows indicate a partial miRNA displacement, and grey dotted arrows indicate which miRNA typology is more expressed in the different urine fractions.

potentiator, whereas the C terminus remains on the mesothelial cell membrane and provides epitopes for immunohistochemistry. However, after subsequent cleavages, it is released as a mesothelin-related peptide [67]. Mesothelin has been studied in both serum and pleural effusion, but it has not demonstrated a high accuracy for MPM diagnosis in either case [67, 68].

Early-stage therapy relies on surgery, but less than 10% of patients are eligible for this therapeutic option. In the advanced stages, chemotherapy with novel antifolates combined with cisplatin offers a slight survival advantage. Early diagnosis and patient stratification to select the best therapy approach in each case are necessary to increase the overall survival of mesothelioma patients [60].

As evidenced in **Table 5**, the main goal of studies of miRNAs in biological fluids has been early diagnosis. miR-126 exhibits the strongest correlation with malignant pleural mesothelioma; specifically, it is downregulated in patient sera [60, 69]. miR-125 may distinguish mesothelioma patients from healthy individuals, but it is not downregulated in lung cancer patients. Therefore, it is also interesting as a marker to differentiate mesothelioma from lung cancer [69].

Table 7. Extracellular miRNAs associated with bladder cancer. MiRNAs were isolated in blood, voided urine, urinary sediment or supernatant. MiRNA identity isdifferent according to the type of fraction where they are detected. miRNAs wereused as diagnostic and prognostic biomarker

miRNA	Liquid	Expression	Use	Number of exa mined subjects	Reference
miR-10b	Urine (Sediment)	Overexpression	Diagnosis	368	[80]
miR-25	Plasma	Underexpression	Diagnosis	48	[76]
miR-27a-3p	Serum	Underexpression	Early Diagnosis; Prognosis	490	[180]
miR-29c	Urine (Sediment)	Overexpression	Diagnosis	368	[80]
miR-30a-5p	Serum	Overexpression	Early Diagnosis	490	[180]
miR-33b	Plasma	Underexpression	Diagnosis; Prognosis	48	[76]
miR-92a	Plasma	Underexpression	Diagnosis; Prognosis	48	[76]
miR-92b	Plasma	Underexpression	Diagnosis; Prognosis	48	[76]
miR-96	Urine (Voided)	Overexpression	Diagnosis	368	[80]
miR-96	Urine (Voided)	Overexpression	Diagnosis, Prognosis; Follow Up	21	[81]
miR-96	Urine (Voided)	Overexpression	Diagnosis, Prognosis; Follow Up	78	[79]
miR-99a	Urine (Supernatant)	Underexpression	Diagnosis, Prognosis	41	[78]
miR-106b	Urine (Supernatant)	Overexpression	Diagnosis	190	[181]
miR-125b	Urine (Supernatant)	Underexpression	Diagnosis, Prognosis	41	[78]
miR-125b	Urine (Voided)	Overexpression	Diagnosis	21	[81]
miR-126	Urine (Voided)	Overexpression	Diagnosis	47	[82]
miR-148b	Plasma	Overexpression	Early Diagnosis	48	[76]
miR-148b	Serum	Overexpression	Early Diagnosis	490	[180]
miR-152	Serum	Overexpression	Diagnosis And Prognosis	490	[180]
miR-152	Urine (Voided)	Overexpression	Diagnosis	47	[82]
miR-155	Urine (Sediment)	Underexpression	Diagnosis	75	[182]
miR-15b-5p	Serum	Overexpression	Early Diagnosis	490	[180]
miR-183	Urine (Voided)	Overexpression	Prognosis	78	[79]
miR-192	Urine (Sediment)	Underexpression	Diagnosis	75	[182]
miR-200	Urine (Sediment)	Underexpression	Diagnosis	75	[182]
miR-200b	Plasma	Overexpression	Diagnosis	48	[76]
miR-210	Urine (Sediment)	Overexpression	Diagnosis	368	[80]
miR-302	Plasma	Underexpression	Diagnosis; Prognosis	48	[76]
miR-3187-3p	Serum	Underexpression	Early Diagnosis; Prognosis	490	[180]
miR-487	Plasma	Overexpression	Diagnosis	48	[76]
miR-541	Plasma	Overexpression	Diagnosis	48	[76]
miR-566	Plasma	Overexpression	Diagnosis	48	[76]

Another interesting miRNA is miR-34b/c. The methylation of this miRNA in patient sera, but not its expression in the blood, correlates with mesothelioma [62].

To increase the sensitivity and specificity of miRNAs for the diagnosis of mesothelioma, miRNA panels have been proposed (**Table 6**) associating the expression of different miRNAs through themselves [61] and other biomarkers, such as small mesothelioma-related peptides [60, 70]. Lamberti divided subjects into two categories based on their miRNA signatures and analyzed the differential expression profiles of miRNAs in the serum to predict prognosis and stage of the tumor and to assess the histotypes of mesothelioma. This particular panel is interesting because it may be used not only for early diagnosis but also to recognize different types of mesothelioma, which facilitates individualized therapy.

Table 8. Panels of extracellular miRNAs used as biomarker in bladder cancer. Multiples miRNAs have been analyzed together to increase their sensitivity and the specificity as biomarker

MiRNA panel	Use	Fluid	Reference
miR-125b; miR-99a	Diagnosis, Prognosis	Urine (Supernatant)	[78]
miR-152; miR-148b-3p; miR-3187-3p; miR-15b-5; miR-27a-3p; miR-30a-5p	Early Diagnosis	Serum	[180]
miR-210; miR-10b; miR-29c	Diagnosis	Urine (Sediment)	[80]

miRNAs are also differentially expressed in pleural effusion [18], and their analysis is complementary to cytology evaluation to differentiate MPM from benign changes due to asbestos [71, 72].

Urinary cancer

Bladder cancer is an adenocarcinoma derived from the transitional epithelium of this organ. It is the most common urological cancer [73] and the fifth most common tumor worldwide [74]. Despite early therapy approaches, urothelial carcinoma continues to cause significant mortality [75].

Most of these malignancies do not invade the muscle but frequently recur. Approximately 70% of patients are initially diagnosed with non-muscle-invasive bladder cancer, but as many as 50-70% of these tumors will recur, and approximately 10-20% will progress to muscle-invasive disease [76].

The gold standard for bladder cancer diagnosis is biopsy via cystoscopy, a painful, costly and invasive type of endoscopy [77]. Non-invasive methods are being analyzed, such as urinary cytology and serological and urinary biomarkers, but they all have low sensitivity and specificity and consequently cannot replace cystoscopy [12].

Due to the high incidence and frequent recurrences of bladder cancer, effective diagnostic and disease-monitoring tools are essential for the clinical management of these patients [78].

Despite the tendency for a relatively indolent course, non-muscle-invasive bladder cancer substantially impacts healthcare due to the costs incurred from frequent cystoscopic surveillance [76].

miRNAs have been studied in the serum and urine. Most studied miRNAs were identified in the urine, and their specificities have been demonstrated in different studies [12, 79].

As shown in **Figure 2**, miRNAs found in voided urine, urinary sediment and the supernatant significantly differ and vary in specificity for bladder cancer [12]. These differences may be associated with the presence of renal tubular cells, urothelial cells, lymphocytes and erythrocytes in urinary sediment, whereas the supernatant contains miRNAs in microvesicles filtered by the glomeruli [12]. A meta-analysis conducted in 2015 of studies of urinary miRNAs found that supernatant miRNAs are more specific than other miRNAs for bladder cancer [12].

miR-96 was overexpressed in the cells of several tumors, such as breast cancer, bladder cancer, testicular cancer and lymphoma [80]. This miRNA was also overexpressed in the urine of bladder-cancer patients and was associated with a poor prognosis; specifically, its concentration directly correlated with the TNM stage [79-81]. miR-96 expression decreases after therapeutic surgery and is consequently of interest as a marker of recurrence [80].

Moreover, miR-129 is also frequently overexpressed in cancer cells, but its concentration in the urine of bladder cancer patients is not significantly altered [80], confirming that not all typical cancer miRNAs undergo significant changes in biological fluids.

As reported in **Table 7**, different miRNAs are associated with bladder cancers and have been tested as early diagnostic markers, demonstrating specificities and sensitivities higher than those of other markers [78, 81, 82]. Some of these miRNAs have been associated with tumor stage, such as miR-96 and miR-183 in voided urine, whose expression levels directly correlate with TNM stage [79]. Furthermore, the downregulation of other miRNAs, such as miR-125b in the urine supernatant, has been associated with tumor grade [78].

Different combinations and panels have also been proposed (**Table 8**) to increase the accuracy of this class of fluid biomarkers. For exam-

ple, Eissa simultaneously evaluated miR-210, miR-10b, and miR-29c. The analysis of this miRNA combination exhibited a sensitivity of 95.2%, and this sensitivity was even higher for early-stage bladder cancer [80].

miRNAs have also been studied in the circulating blood. For example, miR-141 and miR-639 have been identified in the sera of bladder cancer patients [83]. Other miRNAs have been individuated with altered concentrations in bladder cancer and are significant in diagnosis and prognosis [76].

Studies of miRNAs in bladder cancer have focused on early diagnosis, staging and follow-up, but unlike for lung cancer, miRNAs in liquid biopsies have not been studied to analyze chemoresistance, likely because bladder cancer therapy is based on early surgery.

Prostate cancer

Prostate adenocarcinoma is the second most diagnosed malignancy in men worldwide and is exhibiting an increasing incidence in Asia [84]. Furthermore, prostate cancer is the second most common cause of cancer death worldwide [85].

Early diagnosis is a priority in the treatment of this pathology and is based on the prostate-specific antigen (PSA) in combination with clinical examination and prostate palpation via rectal exploration [74, 86, 87]. Notably, as with many cancer biomarkers, PSA should not be used alone to select patients to undergo biopsy because its specificity is low with a high incidence of false positives [88, 89]. Thus PSA should be mainly used in tertiary prevention [4].

The gold standard for early diagnosis is transrectal ultrasound-guided prostate biopsy, an invasive, complex and painful procedure. The detection rate of prostate biopsy is only 30% in PSA-grey-zone patients (patient with an ambiguous PSA value), because the biologic material obtained is frequently inadequate [90], which necessitates new methodologies for prostate cancer diagnosis [91].

Androgen deprivation therapy, a first-line approach that leads to cancer control and palliation, may be used in the advanced stages of metastasis [45]. After the failure of this therapy, which is characterized by the emergence of a castration-resistant disease, survival is limited despite the use of docetaxel and other chemotherapeutic drugs, but this outcome is extremely variable. In this clinical phase, circulating tumor cell counts are the most important prognostic marker, but this test is expensive. Specifically, neoplastic cells in the blood are counted via immunomagnetic enrichment using epithelial cell adhesion molecule, nuclei are fluorescently labeled, and fluorescent monoclonal antibodies are used to identify leukocytes and epithelial cells using a semi-automated fluorescence-based microscopy system [92-94].

Prostate cancer-related miRNAs have primarily been studied in the circulating blood. miRNA-222 and miR-125b overexpression and miR-103 downregulation have been associated with malignancy and early recurrence [95]. Mihelich proposed an miRNA score based on 14 different miRNAs for prostate cancer diagnosis. This approach has demonstrated an odds ratio (OR) of 5.77 for Gleason 3-4 tumors, which is superior to the OR observed for PSA (5.52) [97].

In addition to serving as a reference standard to compare the accuracy of miRNAs, PSA has also been used as a parallel marker integrated in the statistical analysis. Kachakova analyzed the concentrations of let-7c, miR-30c, miR-141, miR-375, and PSA in the sera of 75 patients to distinguish prostate cancer patients from benign prostatic hyperplasia patients. In this study, the integration of PSA and miRNA serum concentrations increased the sensitivity but not the specificity of the test; the highest specificity was associated with miR-375 analysis [96].

Circulating miRNAs have also been tested as prognostic markers. miR-1290 and miR-375 have been isolated in castration-resistant prostate cancer patients, and their levels inversely correlated with the median survival of patients [45].

Circulating miRNAs have been specifically studied as early outcome biomarkers of docetaxel therapy in particular. The pre-docetaxel-treatment concentrations of miR-200 family members (miR-200a, miR-200b, miR-200c), miR-429, and miR-20a in the blood were associated with therapeutic outcome: a high concentration is indicative of poor prognosis [98].

Table 9. Extracellular miRNAs associated with prostate cancer. MiRNAs were isolated in blood, seminal fluid and urine. When urine are collected, patients undergo prostate massage to increase the presence of prostate biomarker in theurine, including miRNAs. miRNAs were used as diagnostic, staging, and prognosticbiomarker

miRNA	Liquid	Expression	Use	Number of examined subjects	Reference
hsv1-miR-H18	Urine	Overexpression	Diagnosis	1354	[91]
hsv2-miR-H9-5p	Urine	Overexpression	Diagnosis	1354	[91]
Let-7a	Serum	Overexpression	Prognosis; Differential Diagnosis	150	[97]
let-7c	Serum	Overexpression	Diagnosis	86	[96]
miR-16	Serum	Overexpression	Diagnosis; Prognosis; Staging	36	[183]
miR-20a	Plasma	Overexpression	Prognosis; Therapy Outcome	97	[98]
miR-21	Serum	Overexpression	Diagnosis	20	[184]
miR-21	Serum	Overexpression	Staging; Metastasis Detection	24	[42]
miR-24	Serum	Overexpression	Prognosis; Differential Diagnosis	150	[97]
miR-26b	Serum	Overexpression	Prognosis; Differential Diagnosis	150	[97]
miR-30c	Serum	Overexpression	Diagnosis	86	[96]
miR-30c	Serum	Overexpression	Prognosis; Differential Diagnosis	150	[97]
miR-34b	Serum	Overexpression	Diagnosis; Prognosis; Staging	36	[183]
miR-92a	Serum	Overexpression	Diagnosis; Prognosis; Staging	36	[183]
miR-92b	Serum	Overexpression	Diagnosis; Prognosis; Staging	36	[183]
miR-93	Serum	Overexpression	Prognosis; Differential Diagnosis	150	[97]
miR-100	Serum	Overexpression	Prognosis; Differential Diagnosis	150	[97]
miR-103	Serum	Overexpression	Diagnosis; Staging; Prognosis	36	[183]
miR-103	Serum	Overexpression	Prognosis; Differential Diagnosis	150	[97]
miR-103	Serum	Underexpression	Therapy Outcome; Prognosis	99	[95]
miR-106a	Serum	Overexpression	Prognosis; Differential Diagnosis	150	[97]
miR-107	Plasma	Overexpression	Diagnosis	106	[99]
miR-107	Serum	Overexpression	Diagnosis; Staging; Prognosis	36	[183]
miR-107	Serum	Overexpression	Prognosis; Differential Diagnosis	150	[97]
miR-125b	Ejaculate	Overexpression	Diagnosis	152	[108]
miR-125b	Serum	Overexpression	Prognosis; Therapy Outcome	99	[95]
miR-130b	Serum	Overexpression	Prognosis; Differential Diagnosis	150	[97]
miR-141	Serum	Overexpression	Diagnosis	86	[96]
miR-141	Serum	Overexpression	Diagnosis	170	[185]
miR-146a	Serum	Overexpression	Prognosis; Differential Diagnosis	150	[97]
miR-183	Urine (Sediment)	Overexpression	Diagnosis	76	[186]
miR-197	Serum	Overexpression	Diagnosis; Prognosis; Staging	36	[183]
miR-200a	Plasma	Overexpression	Prognosis; Therapy Outcome	97	[98]
miR-200b	Plasma	Overexpression	Prognosis; Therapy Outcome	97	[98]
miR-200c	Ejaculate	Overexpression	Diagnosis	152	[108]
miR-200c	Plasma	Overexpression	Prognosis; Therapy Outcome	97	[98]
miR-205	Urine	Underexpression	Diagnosis	50	[101]
miR-205	Urine (Sediment)	Underexpression	Diagnosis	76	[186]
miR-214	Urine	Underexpression	Diagnosis	50	[101]
miR-221	Serum	Overexpression	Diagnosis	20	[184]
miR-222	Serum	Overexpression	Diagnosis	99	[95]
miR-223	Serum	Overexpression	Prognosis; Differential Diagnosis	150	[97]
miR-328	Serum	Overexpression	Diagnosis; Prognosis; Staging	36	[183]
miR-375	Plasma	Overexpression	Prognosis	100	[45]

miR-375	Serum	Underexpression	Diagnosis	86	[96]
miR-375	Serum	Overexpression	Staging; Metastasis Detection	24	[42]
miR-429	Plasma	Overexpression	Prognosis; Therapy Outcome	97	[98]
miR-451	Serum	Overexpression	Prognosis; Differential Diagnosis	150	[97]
miR-485-3p	Serum	Overexpression	Diagnosis; Prognosis; Staging	36	[183]
miR-486-5p	Serum	Overexpression	Diagnosis; Prognosis; Staging	36	[183]
miR-574	Serum	Overexpression	Staging; Metastasis Detection	24	[42]
miR-574-3p	Plasma	Overexpression	Diagnosis	106	[99]
miR-574-3p	Serum	Overexpression	Diagnosis; Prognosis; Staging	36	[183]
miR-636	Serum	Overexpression	Diagnosis; Prognosis; Staging	36	[183]
miR-640	Serum	Overexpression	Diagnosis; Prognosis; Staging	36	[183]
miR-766	Serum	Overexpression	Diagnosis; Prognosis; Staging	36	[183]
miR-874	Serum	Overexpression	Prognosis; Differential Diagnosis	150	[97]
miR-885.5p	Serum	Overexpression	Diagnosis; Prognosis; Staging	36	[183]
miR-888	Eps	Overexpression	Diagnosis; Prognosis; Staging	56	[102]
miR-889	Urine (Supernatant)	Overexpression	Diagnosis; Prognosis; Staging	56	[102]
miR-1290	Plasma	Overexpression	Prognosis	100	[45]

Table 10. Panels of extracellular miRNAs used as biomarker in prostate cancer. Multiple miRNAs have been analyzed together to increase their sensitivity and specificity as biomarker. In prostate cancer, the most of the studies has includedalso PSA analysis

MiRNA panel	Use	Fluid	Reference
PCA; miR-200c; miR-125b	Diagnosis	Ejaculate	[108]
Let-7c; miR-30c; miR-141; miR-375; PSA	Diagnosis	Serum	[96]
Let-7a: miR-103; miR-107; miR-130b; miR-106a; miR-26b; miR-451; miR-223; miR-93; miR-24; miR-30c; miR-874; miR-100; miR-146a	Prognosis; Differential Diagnosis	Serum	[97]

A lack of changes in miRNA concentration after chemotherapy is important for prognosis. Lin proposed a prognostic model that combines miR-20a post-therapy changes, the presence of a visceral metastasis, the pre-docetaxel hemoglobin level and the pre-docetaxel miR-200b level to predict patient survival [98]. His model, which was tested in 97 patients, exhibits an area under the curve (AUC) of 0.77 [98], which is superior compared to single factors. This finding suggests that miRNA integration with clinical indicators is possible and useful.

miRNAs related to prostate cancer have been researched in different biological fluids, including urine. Urine samples were generally collected after a trans-rectal digital prostatic massage finalized to transfer in urines prostatic antigens, miRNAs included [99]. This procedure increased the detection rate of probable prostate-specific genetic markers in urine [100].

As evidenced in **Table 9**, different human miRNAs have been associated with prostate adenocarcinoma. For example, miR-107 and miR-574-3p expression levels were increased [99], whereas others, such as miR-205 and miR-214, were downregulated in this clinical condition [101].

Urine miRNAs have also been studied as prognostic markers. miR-888 of prostate origin was identified in the urine of primary prostatic cancer patients and has been correlated with high-grade malignancies; consequently, this miRNA indicates a poor prognosis [102].

Viral miRNAs have also been linked to prostate cancer. HPV and Herpes simplex virus (HSV) 2 have been suggested to play roles in prostate cancer development [103]. In a recent meta-analysis of 11 articles [104], HSV 2 infection was associated with prostate cancer risk with an OR of 1.209. In the same study, Kaposi Sarcoma Virus did not demonstrate a correlation with this type of malignancy [104], which suggests that HSV 2 exhibits unique oncogenic potential and justifies an analysis of HSV miRNAs.

Two virus-encoded miRNAs, hsv2-miR-H9-5p and hsv1-miR-H18, are overexpressed in the urine of prostate cancer patients compared with benign prostatic hyperplasia patients. Two other herpetic miRNAs, hsv1-miR-H18 and hsv2-miR-H9-5p, provide better diagnostic performance than the total serum PSA, and serum PSA and urine miR-H9-5p analysis have been proposed as substitutes for transrectal biopsy [91]. Despite the identified correlation between HPV and prostate carcinoma, we have not identified studies that analyzed the correlation between HPV miRNA and this malignancy. Nevertheless, this correlation has been studied in cervical cancer and head and neck cancer [105-107].

miRNA panels have been used to improve biomarker accuracy (**Table 10**). Specifically, miRNAs have been identified in seminal fluid [18], and miR-200c and miR-125b have been isolated in ejaculate and associated with prostate cancer. The combination of their ejaculate concentrations with serum PSA was established as a diagnostic index with a good sensitivity (90%) and a discrete specificity (67%) [108]. Another miRNA panel was tested in the blood serum [96].

Breast cancer

Breast cancer is one of the leading causes of cancer death and one of the most commonly diagnosed tumors in women [109]. Specifically, one in every eight women has been estimated to develop this cancer during her lifetime [110].

An early diagnosis and screening campaign has reduced breast cancer mortality in 40- to 50-year-old women, who are at high risk of developing tumors [111-113]. Cancer diagnosis is based on ultrasound in young women and mammography in the remaining female population, although magnetic resonance is used in specific cases, such as breast implant [111-113].

Mammography is highly specific, not very sensitive, and expensive, and it exposes the patient to ionizing radiation. Therefore, breast self-examination is used instead as a diagnostic method, but it has a low specificity, which increases the numbers of false positives, control mammographies and biopsies, incurs high costs and results in damage and general stress to the patient. Furthermore, breast self-examination has a very low sensitivity and can only detect cancer of already remarkable size. Therefore, breast cancer is often diagnosed late. Echography-guided biopsy is the gold standard of diagnosis [114].

Serological markers, such as Ca15.3 and CEA, are already in use, but they have low specificity and are used for patient follow-up to detect relapse [115]. Occasionally, these markers are also used for prognosis [116].

Therapy is based on surgical approaches supported by adjuvant and neo-adjuvant radiotherapy and chemotherapy. Prognosis is associated with grade, stage and other parameters, such as the absence of the estrogen receptor or the overexpression of epidermal growth factor receptor (HER2) in cancer cells, which is associated with a poor prognosis [117]. HER2 is the target of monoclonal antibodies, such as trastuzumab, a class of molecules used in neoadjuvant therapy. However, tumors ultimately develop resistance within three years [118].

Thus, biomarkers for early diagnosis are lacking, and available biomarkers are only applicable to advanced breast cancer. Accordingly, innovative biomarkers to be used in primary prevention and the selection of high-risk subjects for secondary prevention via mammographic screening are urgently needed.

miRNAs are secreted from breast cancer tumors, likely selectively, into the circulating blood and milk [18], but the most commonly studied liquid, as evidenced in **Table 11**, is the blood [119]. Weber has identified high concentrations of miRNAs in human milk, but these miRNAs have not been studied, likely because breast cancer is rare in lactating women.

One of the first goals of miRNA studies was the early diagnosis of breast cancer based on the identification of altered oligonucleotides in the serum and plasma, including miR-202 [20], miR-205 [120] and miR-409-3p [121]. Some of these miRNAs, i.e., miR-16, miR-21 and miR-451, were significantly reduced in post-surgical samples, implicating them as possible markers for the early diagnosis of relapse [115].

As evidenced in **Table 11**, several miRNAs have been associated with breast cancer in single studies, some of which were based on cohorts of 100 patients [121]. However, the same miRNA was rarely identified as a reliable biomarker in different studies, or miRNAs were

Table 11. Extracellular miRNAs associated with breast cancer. MiRNAs were isolated in blood and sometimes in urine. miRNAs were used as diagnostic, staging, and prognostic biomarker. Some miRNAs are associated with specific types of tumor, such as triple-negative breast cancer, related to drug resistance and poor prognosis

miRNA	Liquid	Expression	Use	Number of examined subjects	Reference
miR.-107	Plasma	Overexpression	Staging; Prognosis	157	[187]
miR-1	Serum	Overexpression	Diagnosis	233	[114]
miR-10b	Serum	Overexpression	Prognosis; Staging	170	[124]
miR-10b	Serum	Overexpression	Diagnosis	71	[122]
miR-10b-5p	Serum	Overexpression	Diagnosis; Stadiaton	149	[123]
miR-16	Plasma	Overexpression	Therapy Outcoma; Staging	157	[187]
miR-16	Serum	Overexpression	Diagnosis	485	[115]
miR-18b	Serum	Overexpression	Prognosis	130	[188]
miR-21	Serum	Overexpression	Diagnosis; Prognosis; Staging; Therapy Outcome	71	[122]
miR-21	Serum	Overexpression	Therapy Outcome	146	[125]
miR-21	Serum	Overexpression	Diagnosis	146	[115]
miR-21	Serum	Overexpression	Diagnosis; Prognosis; Staging; Therapy Outcome	90	[189]
miR-21	Serum	Overexpression	Diagnosis; Prognosis; Staging; Therapy Outcome	213	[167]
miR-21	Serum	Overexpression	Diagnosis; Prognosis; Staging; Therapy Outcome	52	[190]
miR-21	Urine (Supernatant)	Underexpression	Diagnosis	48	[191]
miR-27a	Plasma	Overexpression	Therapy Outcome	157	[187]
miR-34a	Serum	Underexpression	Staging; Prognosis	170	[124]
miR-92a	Serum	Overexpression	Diagnosis	233	[114]
miR-101	Serum	Overexpression	Diagnosis; Prognosis	277	[127]
miR-103	Serum	Overexpression	Prognosis	130	[188]
miR-107	Serum	Overexpression	Prognosis	130	[188]
miR-125b	Serum	Overexpression	Diagnosis	71	[122]
miR-125b	Urine (Supernatant)	Underexpression	Diagnosis	48	[191]
miR-130a	Plasma	Overexpression	Staging; Prognosis	157	[187]
miR-132	Plasma	Overexpression	Therapy Outcome	157	[187]
miR-133a	Serum	Overexpression	Diagnosis	233	[114]
miR-133b	Serum	Overexpression	Diagnosis	233	[114]
miR-145	Serum	Overexpression	Diagnosis	71	[122]
miR-145	Serum	Underexpression	Diagnosis	485	[115]
miR-146a	Plasma	Overexpression	Staging; Prognosis	157	[187]
miR-148b	Serum	Overexpression	Diagnosis	207	[121]
miR-148b-3p	Serum	Underexpression	Diagnosis	149	[123]
miR-155	Serum	Overexpression	Staging; Prognosis	170	[124]
miR-155	Serum	Overexpression	Diagnosis; Staging; Prognosis	71	[122]
miR-155	Serum	Underexpression	Therapy Outcome	158	[192]
miR-155	Urine (Supernatant)	Overexpression	Diagnosis	48	[191]
miR-181a-5p	Serum	Overexpression	Diagnosis	207	[193]
miR-191	Serum	Overexpression	Diagnosis	71	[122]
miR-195	Serum	Overexpression	Staging	170	[124]
miR-199a-5p	Plasma	Overexpression	Prognosis	357	[126]
miR-202	Serum	Overexpression	Diagnosis	153	[20]
miR-205	Serum	Underexpression	Diagnosis	151	[120]
miR-210	Plasma	Overexpression	Prognosis; Staging; Therapy Outcome	170	[84]
miR-210	Plasma	Overexpression	Therapy Outcome	146	[125]
miR-373	Serum	Overexpression	Prognosis	277	[127]
miR-373	Serum	Overexpression	Therapy Outcome	146	[125]
miR-376c	Serum	Overexpression	Diagnosis	207	[121]

miR-382	Serum	Overexpression	Diagnosis	71	[122]
miR-409-3p	Serum	Overexpression	Diagnosis	207	[121]
miR-451	Serum	Overexpression	Diagnosis	485	[115]
miR-451	Urine (Supernatant)	Underexpression	Diagnosis	48	[191]
miR-652	Serum	Overexpression	Prognosis	130	[188]
miR-652-3p	Serum	Underexpression	Diagnosis	149	[123]
miR-718	Serum	Underexpression	Diagnosis	153	[20]
miR-801	Serum	Overexpression	Diagnosis	207	[121]

Table 12. Panels of extracellular miRNAs used as biomarker in breast cancer. Multiples miRNAs have been analyzed together to increase their sensitivity and specificity as biomarker

Panel	Use	Fluid	Reference
miR-1; miR-92a; miR-133a; miR-133b	DiagnosIs	Serum	[114]
miR-18b; miR-103; miR-107; miR-652	Prognosis	Serum	[188]
miR-145; miR-155; miR-382	Diagnosis	Serum	[122]
miR-145; miR-451	Diagnosis	Serum	[115]
miR-148b; miR-409-3p; miR-801	Diagnosis	Serum	[121]

demonstrated to be more accurate than mammography. Therefore, miRNAs have been compared in panels, which increased their sensitivity and specificity above 95% in some studies of miRNA profiles [122]. **Table 12** reports the miRNA associations proposed in the analyzed studies.

miRNAs were also studied as prognostic markers. miR-10b has been associated with advanced-stage breast cancer and consequently with a worse prognosis [123]. miR-10b and miR-155 were associated with lymph node and distant metastases, making them interesting as both prognostic markers and for early detection of metastasis [124]. Several forms of miR-21 have been associated with a worsening prognosis but not with reduced disease-free survival and therapy response [125].

Increases in miR-199a-5p, miR-101 and miR-373 have been associated with triple-negative breast cancer, a type of breast tumor characterized by the absence of receptor for estrogen (ER), progesterone (PR) and human epithelial growth factor (HER) [126, 127]. Triple-negative breast cancer is resistant to biological drugs, such as trastuzumab, and has a poor prognosis because it is usually highly aggressive [126].

miRNAs may also be used to evaluate therapeutic outcome. Specifically, chemotherapy increases the serologic concentration of miRNAs in breast cancer patients [84, 125], most likely due to massive cancer cell death. miR-210 overexpression after chemotherapy has been associated with a non-complete response to therapy [125]. Moreover, the serological expression of miR-21, miR-210 and miR-373 has been studied in patients treated with neoadjuvant chemotherapy with trastuzumab. Specifically, increases in these miRNA expression levels were associated with a good response to therapy, identifying them as markers that can be used to monitor therapy [84, 125].

Hematologic malignancies

Hematologic malignancies are a group of pathologies characterized by the uncontrolled proliferation of circulating blood cells or their precursors. This peculiar category of malignancies is classified based on the type of cell that aberrantly proliferates and the distribution of clones - in masses or diffuse throughout the blood [128, 129].

miRNAs have been associated with the control of hematopoietic cell replication and maturation. Therefore, they have been studied as disease status biomarkers and therapeutic targets [130].

Diffuse large B-cell lymphoma is the most studied form of lymphoma and constitutes 40% of adult non-Hodgkin lymphomas [2]. This group of lymphomas is clinically very heterogeneous, especially because it can develop at different sites of the body, which affects prognosis and therapeutic options [2, 131]. Moreover, this malignancy frequently relapses in the testes and brain [2].

Therapy is based on chemotherapy, which was revolutionized by anti-CD20 antibodies (e.g., rituximab), radiotherapy, and eventually a surgical approach [131].

Table 13. Extracellular miRNAs associated with hematologic malignances. MiRNAs were isolated in blood. miRNAs were used as diagnostic and prognostic biomarker

miRNA	Liquid	Expression	Use	Tumor	Number of examined subjects	Reference
miR-10a-5p	Serum	Overexpression	Prognosis; Follow Up	Adult Acute Myelod Leukemia	275	[135]
miR-10a-5p	Serum	Overexpression	Prognosis; Follow Up	Adult Acute Myelod Leukemia	275	[8]
miR-15a	Serum	Overexpression	Diagnosis	Diffuse Large B Cell Lymphoma	75	[131]
miR-16-1	Serum	Overexpression	Diagnosis	Diffuse Large B Cell Lymphoma	75	[131]
miR-20a	Plasma	Overexpression	Diagnosis; Prognosis	Chronic Linfatic Leukemia	48	[139]
miR-21	Serum	Overexpression	Prognosis	Large B-Cell Lymphoma	75	[131]
miR-21	Serum	Overexpression	Prognosis	Large B-Cell Lymphoma	103	[130]
miR-21	Serum	OverexpressiOn	Prognosis	Large B-Cell Lymphoma	121	[132]
miR-29c	Serum	Overexpression	Diagnosis	Large B-Cell Lymphoma	75	[131]
miR-34a	Serum	Underexpression	Diagnosis	Large B-Cell Lymphoma	75	[131]
miR-93-5p	Serum	Overexpression	Prognosis	Adult Acute Myelod Leukemia	275	[135]
miR-129-5p	Serum	Overexpression	Prognosis	Adult Acute Myelod Leukemia	275	[135]
miR-150	Serum	Overexpression	Prognosis	Chronic Linfatic Leukemia	540	[138]
MiR-155	Serum	Overexpression	Diagnosis	Large B-Cell Lymphoma	75	[131]
MiR-155	Serum	Overexpression	Prognosis	Large B-Cell Lymphoma	103	[130]
miR-155-5p	Serum	Overexpression	Prognosis	Adult Acute Myelod Leukemia	275	[135]
miR-181b-5p	Serum	Overexpression	Prognosis	Adult Acute Myelod Leukemia	275	[135]
miR-195	Plasma	Overexpression	Diagnosis	Chronic Linfatic Leukemia	48	[139]
miR-199b-5p	Plasma	Overexpression	Diagnosis; Therapy Outcome	Acute Myelod Leukemia	18	[136]
miR-210	Serum	Overexpression	Prognosis	Large B-Cell Lymphoma	103	[130]
miR-301b	Plasma	Overexpression	Diagnosis; Therapy Outcome	Acute Myelod Leukemia	18	[136]
miR-320d	Serum	Overexpression	Prognosis	Adult Acute Myelod Leukemia	275	[135]
miR-326	Plasma	Overexpression	Diagnosis; Therapy Outcome	Acute Myelod Leukemia	18	[136]
miR-335	Serum	Overexpression	Diagnosis; Therapy Outcome	Pediatric Acute Myelod Leukemia	156	[134]
miR-361-5p	Plasma	Overexpression	Diagnosis; Therapy Outcome	Acute Myelod Leukemia	18	[136]
miR-625	Plasma	Overexpression	Diagnosis; Therapy Outcome	Acute Myelod Leukemia	18	[136]
miR-638	Plasma	Overexpression	Prognosis; Follow Up	Chronic Linfatic Leukemia	48	[139]
miR-655	Plasma	Overexpression	Diagnosis; Therapy Outcome	Acute Myelod Leukemia	18	[136]

The use of miRNAs for the early diagnosis of these diseases has been studied since 2007, especially because the only diagnostic approach in patients bearing metastases in the central nervous system is biopsy, a high-risk procedure that is often complicated by hemorrhages [2] (**Table 13**).

Lawrie at al. searched the sera of 60 patients and 40 controls for a common miRNA pattern, identifying three possible candidate oncologic markers: miR-155, miR-210 and miR-21 [130]. The most interesting of these markers was miR-21, an miRNA linked to anti-proliferation and involved in several forms of tumors that are associated with more favorable clinical outcomes [130].

Chen's group also studied miR-21 and demonstrated that its serum level correlated with the diffuse large B-cell lymphoma subtype and its clinical stage, confirming Lawrie's studies [132]. miR-21 was also demonstrated to be independent of other prognostic parameters, such as age and gender.

The concentrations of miR-16-1, miR-21, miR-29 and mir-155 are elevated in the sera of patients bearing diffuse large B-cell lymphoma, and these levels are not significantly associated with other factors, such as gender, age, stage, and extranodal involvement. Thus, these miRNAs have been proposed as independent prognostic and diagnostic biomarkers [131].

Due to their diagnostic challenges, primary central nervous system lymphomas have been extensively studied. The primary site of this malignancy is the brain, and it exhibits an ambiguous symptomatology that requires a complex therapeutic approach, which is mainly based on methotrexate [133].

Differentiating primary central nervous system lymphomas from metastasis, glioblastoma and central nervous system inflammation consti-

tutes another challenge. miR-21 is significantly overexpressed in the sera of patients with primary central nervous lymphoma but not in healthy patients and central nervous system inflammatory patients. Mir-21 is a common oncological biomarker that was upregulated in the sera of glioblastoma patients. However, its concentration was significantly higher in primary central nervous lymphoma [133].

Leukemia constitutes another type of hematologic malignancy. Leukemia refers to a heterogenic group of diseases characterized by the uncontrolled proliferation of hematopoietic precursors and their invasion of the blood. Leukemia is classified based on the cell type and clinical course [134].

Acute myeloid leukemia is characterized by granulocytic, monocytic, megakaryocytic, or rarely, erythroid blast cell proliferation, which causes hematopoietic insufficiency. This subtype comprises 30% of pediatric leukemia deaths and is characterized by a 5-year disease-free survival rate of 50% in pediatric patients. Acute myeloid leukemia is also common in adults [135].

This pathology is treated with chemotherapy and allogenic stem cell transplantation, especially in pediatric cases [134]. However, therapy approach is influenced by the FAB classification, which distinguishes eight categories based on cytogenetic information, each of which features a unique prognosis and gene alterations [135]. In this context, miRNAs have been specifically studied as prognostic factors that may predict therapy response, FAB classification, and aberrant mutations.

The miR-335 level in the serum is associated with M7 acute myeloid leukemia and unfavorable karyotypes, making this miRNA a candidate independent prognostic factor [134]. Furthermore, miR-155-5p was also overexpressed in patient sera, and this overexpression was associated with adverse clinical outcome and FLT3 internal tandem duplication. FLT3, also known as CD135, is a tyrosine kinase class III protein expressed on the surfaces of different hematopoietic progenitor cells [135].

Recently, miRNAs have also been studied as markers of therapy response. Specifically, the acute myeloid leukemia status influenced the miR-10a-5p serum concentration, making it a good marker for follow-up [8]. A pilot study conducted by Koutova identified six miRNAs that are overexpressed in the plasma of patients before therapy and are not detectable in healthy individuals [136].

Hornick xeno-transplanted human acute myeloid leukemia cells into immunodeficient mice and detected exosomes containing miR-150, miR-155, and miR-1246, miRNAs that are correlated with relapse. Therefore these miRNAs may be early markers of recurrence [137].

miRNAs have also been studied in chronic lymphocytic leukemia (CLL), the most common type of leukemia encountered in developed countries. In this malignancy, CD5+ B lymphocyte clones invade the blood, bone marrow and lymph nodes [138]. This malignancy is characterized by a heterogeneous clinical outcome influenced by several factors, such as age, clinical stage and specific mutations [138]. Furthermore, chemotherapy can only slow the progression of this pathology and usually fails to induce full recovery [139].

Moussay demonstrated that miR-195 and miR-20a overexpression was able to distinguish healthy patients from CLL patients. Therefore, these miRNAs may be used as diagnostic markers. They also demonstrated that miR-20a was significantly correlated with disease severity in a manner similar to that of ZAP-70, a protein expressed on the T cell surface as part of the T cell receptor, which is used as a marker of CLL prognosis [139].

Stomatopoulos recently conducted a study of miR-150 expression in leukemic cells and patient sera. He demonstrated the correlation of this miRNA with prognosis severity, but intracellular miR-150 inversely correlated with leukemia severity, whereas the extra-cellular serum miR-150 levels directly related to leukemia severity. However, the serum and cellular miR-150 expression levels did not correlate and were independent of each other [138].

Neurological cancer

The most frequent brain malignancies are metastases, especially from lung and breast cancer, whereas the most frequent primary brain malignancies are glioblastoma and cerebral B lymphomas [23].

Table 14. Extracellular miRNAs associated with neurological cancer. MiRNAs were isolated in blood and CSF. miRNAs were used for prognosis and differential diagnosis. The most common tumors involved in differential diagnosis of cerebral metastasis are breast cancer and lung cancer and miR-200a and miR-200b were able to distinguish between these two malignances

miRNA	Liquid	Expression	Use	Tumor	Number of examined subjects	Reference
miR-9-3p	Serum	Overexpression	Diagnosis	Neuroblastoma	53	[151]
miR-10b	CSF	Overexpression	Differential Diagnosis	Glioblastoma; Brain Metastasis	118	[23]
miR-15b	CSF	Overexpression	Diagnosis	Primary Cerebral Large Diffuse B-Lymphoma	53	[142]
miR-15b	CSF	Overexpression	Diagnosis	Glioma	50	[145]
miR-19b	CSF	Overexpression	Diagnosis	Primary Cerebral Large Diffuse B-Lymphoma	53	[142]
miR-21	CSF	Overexpression	Diagnosis	Glioma	26	[140]
miR-21	CSF	Overexpression	Diagnosis	Primary Cerebral Large Diffuse B-Lymphoma	53	[142]
miR-21	CSF	Overexpression	Diagnosis	Glioma	50	[142]
miR-21	CSF	Overexpression	Differential Diagnosis	Glioblastoma; Brain Metastasis	118	[23]
miR-21	Plasma	Overexpression	Diagnosis	Glioblastoma	60	144
miR-21	Serum	Overexpression	Diagnosis; Prognosis	Primary Cerebral Large Diffuse B-Lymphoma	178	[133]
miR-92a	CSF	Overexpression	Diagnosis	Primary Cerebral Large Diffuse B-Lymphoma	53	[142]
miR-124-3p	Serum	Overexpression	Diagnosis	Neuroblastoma	53	[151]
miR-128	Plasma	Underexpression	Diagnosis; Prognosis	Glioblastoma	60	144
miR-129c	Serum	Overexpression	Diagnosis	Neuroblastoma	53	[151]
miR-141	CSF	Overexpression	Differential Diagnosis	Glioblastoma; Brain Metastasis	118	[23]
miR-200a	CSF	Overexpression	Differential Diagnosis	Glioblastoma; Brain Metastasis	118	[23]
miR-200b	CSF	Overexpression	Differential Diagnosis	Glioblastoma; Brain Metastasis	118	[23]
miR-200c	CSF	Overexpression	Differential Diagnosis	Glioblastoma; Brain Metastasis	118	[23]
miR-218-5p	Serum	Overexpression	Diagnosis	Neuroblastoma	53	[151]
miR-342-3p	Plasma	Underexpression	Diagnosis; Prognosis	Glioblastoma	60	144
miR-490-5p	Serum	Overexpression	Diagnosis	Neuroblastoma	53	[151]
miR-1538	Serum	Overexpression	Diagnosis	Neuroblastoma	53	[151]

The diagnosis of these malignancies is based on clinical examination and magnetic resonance imaging [140], but the gold standard is biopsy, which is complicated by hemorrhages and neurological damage [1, 141] and cannot be used to assess disease progression in real time.

Attempts have been made to analyze the CSF, which is less invasive than biopsy, especially during the 1st year of life. However, the analyzed markers did not exhibit acceptable specificity and sensitivity [142].

Glioblastoma is the most frequent and deadliest form of primary brain neoplasm in adults [143]. The standard approach is based on surgical resection with adjuvant chemotherapy (nitrosourea is the most commonly used drug) and radiotherapy, but the median survival period is approximately 14 months [144].

Glioblastoma secretes extracellular vesicles, which contain genetic material with a specific pattern (especially miRNAs), into both the systemic blood circulation (detectable in the serum and in the plasma) and the CSF [140].

Therefore, miRNAs have been studied in both the blood and CSF, but these studies yielded different results. miR-21 is detectable both in the serum and CSF, but its level in the blood does not correlate with glioblastoma occurrence, likely because extra-vesicles from hematopoietic cells act as a confounding factor. Therefore, this biomarker exhibits poor blood specificity [140]. Mir-21 was also analyzed in the CSF, and its overexpression in exosomes correlated with glioblastoma with high specificity and sensitivity. Furthermore, clinical resection was associated with a decrease in miR-21 expression [140].

miR-21 is one of the most studied miRNAs in the context of glioblastoma. miR-10b has been associated with all of the most common types of brain malignancies [23].

MiRNAs expressed only in metastases and not in primary brain tumors have been studied for

use in differential diagnosis (**Table 14**). Four interesting candidates, miR-200a, miR-200b, miR-200c and miR-141, are typical of the breast and lung and only altered in this type of cancer. These miRNAs were also isolated from the CSF of patients with brain metastases, but they are not overexpressed in the CSF of glioblastoma patients [23].

miRNAs have also been studied in other types of brain cancer, such as gliomas. For example, Baraniskin demonstrated that analyzing miR-21, miR-10b, miR-200 and miR-125b in the CSF differentiates healthy patients from glioma patients [145].

miR-21 was also studied in the plasma and associated with gliomas. High levels of miR-128 and miR-342-3p expression in the plasma have been associated with glioblastoma, discriminating healthy individuals from patients with glioma with high sensitivity and specificity [146].

Primary diffuse large B-cell lymphoma, whose primary site is the brain, presents a diagnostic and therapeutic challenge in the field of cerebral malignancies. The diagnosis of this pathology is complex because radiography and responsiveness to corticosteroids cannot be used to distinguish lymphoma from inflammatory central nervous system disease. Cerebral biopsy can distinguish between these pathologies, but it cannot be performed in all cases [1].

Primary diffuse large B-cell lymphoma is highly aggressive and, without early diagnosis, rapidly leads to death [141]. The first therapeutic approach is based on methotrexate polychemotherapy [133]. MiR-21 was isolated from patient serum and associated with primary diffuse large B-cell lymphoma, identifying this miRNA as a diagnostic marker. Specifically, it can be used to distinguish primary diffuse large B-cell lymphoma from central nervous inflammatory disease [133]. The overexpression of miR-21 in the serum is associated with a poor prognosis [133], and it is overexpressed in the CSF of patients bearing primary diffuse large B-cell lymphoma.

Two additional miRNAs associated with this type of lymphoma are miR-19b and miR-92a. Baraniskin proposed a diagnostic tree based on the expression of these two miRNAs and miR-21 in the CSF, and this panel demonstrated good specificity and sensitivity [142].

Another important cerebral malignancy is neuroblastoma, an extracranial tumor that is considered to be an embryonic cancer derived from the sympathetic nervous system [147]. It occurs in children under 5 years old [148], its incidence is 10.5 cases per million in children and it represents 12-15% of all deaths due to cancer in children [149, 150].

Several miRNAs, such as let-7 [147] and miR-497 [150], have been associated with this malignancy, but we found one only study of liquid biopsy for this malignancy. Specifically, Murray analyzed changes in a panel of 6 miRNAs in an effort to distinguish different pediatric tumors [151]. This panel was able to distinguish neuroblastoma patients from hepatoblastoma and Wilms' tumor patients [151]. Furthermore, this panel also detected MYCN-amplified high-risk neuroblastoma [151]. MYCN is a member of the MYC family, and its overexpression in neuroblastoma is associated with a poor prognosis [152].

Despite its clinical relevance, the low number of studies dedicated to neuroblastoma can be attributed to the difficulties associated with conducting studies in children.

Conclusions

miRNAs detected in liquid biopsies have demonstrated good specificity and sensitivity, superior to those of other markers. Therefore, they may be used to conduct low-cost and non-invasive tests. However, the use of miRNA as cancer biomarkers is subject to limitations. Studies of the use of miRNAs as diagnostic tools have indicated a wide range of sensitivity and specificity values and have indicated different miRNAs as biomarkers for the same type of tumor. This variation may be the result of differences in the types of miRNAs profiled and the specimens tested [12], i.e., the ethnicities of the cohorts, the use of endogenous controls, the source of miRNAs (whole blood, serum and plasma) and the methods of detection can vary significantly between studies. Accordingly, there is an urgent need to standardize procedures to further develop the use of miRNAs as cancer biomarkers.

Another obstacle in the way of obtaining useful miRNA profiles from serum and plasma samples is that physiological and pathologic pro-

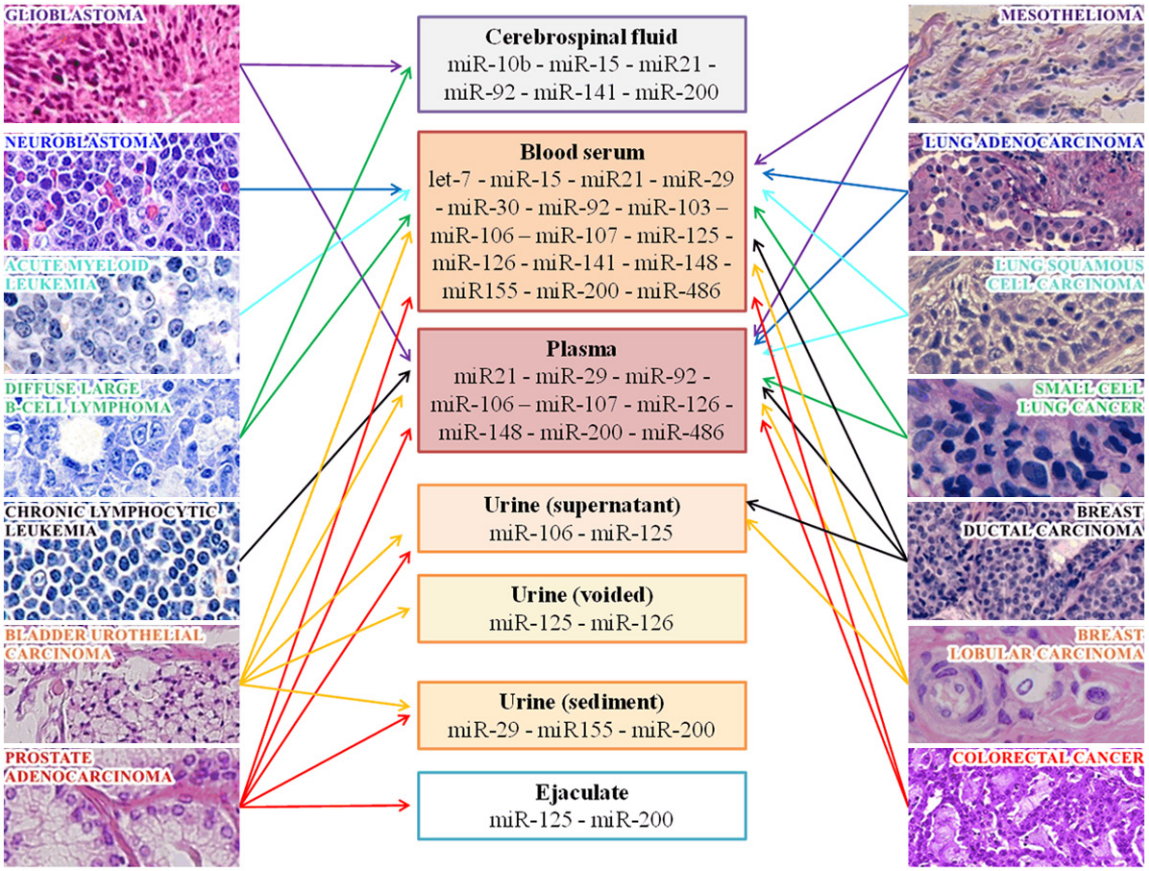

Figure 3. Release of extracellular miRNA in body fluids from various cancer types.

cesses other than cancer may alter miRNA expression profiles [13]. Most studies have demonstrated that miRNAs can distinguish healthy people from cancer patients. However, these studies frequently did not test the same miRNAs in patients with other diseases. Thus, the data were insufficient to establish the specificity of this biomarker. Furthermore, lifestyle factors, including cigarette smoking [56], diet [153] and physical activity [154], remarkably affect the miRNA profile, which limits the specificity of these biomarkers for cancer detection [17].

Nevertheless, as previously discussed, several extracellular miRNAs are associated with various tumor types (**Figure 3**), mainly including miR-21, miR-10b, miR126, miR-155, the miR-30 family, and miR-92. MiR-21 is involved in apoptosis signaling pathways, such as AKT and MAPK [155, 156]. miR-10b is a transcriptional factor involved in embryonic development [157] and in the S phase and mitotic transition, mediating the expression of several transcription factors, such as E2F1 [158]. mir-126 can act as either an oncogene or an oncosuppressor in different tumors, but its true function is not clear [69, 159]. However, it likely interacts with Kloth proteins, which regulate growth factor signaling pathways [160]. miR-155 is involved in myeloproliferation and blocks the differentiation and regulation network (e.g., FLT3, Fms-related tyrosine kinase 3) of hematopoietic cells [161]. The miR-30 family is involved in the transcription of ITGB3 (integrin beta-3) and the ubiquitin-conjugating enzyme E2, which plays roles in transcriptional regulation, nuclear trafficking and protein stability [162]. miR-92 is involved in the expression of estrogen receptor b and mucin 16 (CA-125) [163].

The study of blood-specific miRNAs in the plasma or serum has also been debated. miRNAs are identifiable in both the plasma and serum and exhibit differences in both fluids, but studies cannot univocally identify the best fluid to

study. Generally, plasma miRNAs are more accurate [164], but exceptions have been identified. For example, serum miRNAs have demonstrated higher accuracy in colorectal cancer [11]. The reasons for these differences are not clear but may be related to blood cell miRNAs and hemolysis [164]. Indeed, blood cells entrapped in clots may be damaged and could consequently release intracellular miRNAs, an occurrence that generates artifacts in the interpretation of extracellular miRNAs in the serum.

In conclusion, miRNAs are very interesting as cancer biomarkers, but additional studies, especially meta-analyses of pooled data from different studies, are needed to validate the efficacy of miRNAs and to identify reliable miRNA panels to be used as cancer biomarkers.

Disclosure of conflict of interest

None.

Abbreviations

CSF, cerebrospinal fluid; CT, Computed Tomography; miRNA, microRNA; PSA, Prostatic Specific Antigen.

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