Review

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The circulating miRNAs as diagnostic and prognostic markers

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Abstract: A large portion of the human genome transcribes RNA sequences that do not code for any proteins. The first of these sequences was identified in 1993, and the best known noncoding RNAs are microRNA (miRNAs). It is now fully established that miRNAs regulate approximately 30% of the known genes that codify proteins. miRNAs are involved in several biological processes, like cell proliferation, differentiation, apoptosis and metastatization. These RNA products regulate gene expression at the post-transcriptional level, modulating or inhibiting protein expression by interacting with specific sequences of mRNAs. Mature miRNAs can be detected in blood plasma, serum and also in a wide variety of biological fluids. They can be found associated with proteins, lipids as well as enclosed in exosome vesicles. We know that circulating miRNAs (C-miRNAs) can regulate several key cellular processes in tissues different from the production site. C-miRNAs behave as endogenous mediators of RNA translation, and an extraordinary knowledge on their function has been obtained in the last years. They can be secreted in different tissue cells and associated with specific pathological conditions. Significant evidence indicates that the initiation and progression of several pathologies are "highlighted" by the presence of specific C-miRNAs, underlining their potential diagnostic relevance as clinical biomarkers. Here we review the current literature on the possible use of this new class of molecules as clinical biomarkers of diseases.

Keywords: biomarkers; circulating miR; circulating miRNAs (C-miRNAs).

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Introduction

microRNA biogenesis

The central dogma of molecular biology has been used for years to describe the flow of genetic information. The idea was that the information flows from deoxyribonucleic acid (DNA), to ribonucleic acid (RNA), to proteins, and that the latter provide the structural and regulatory functions of cells and tissues [1].

In the past decade a number of reports have demonstrated transfers of information not explicitly covered by the dogma, such as the discovery of prion proteins [2, 3] and of a large portion of the genome transcribed to RNA sequences that do not code for any proteins. These groups of RNA are constituted by microRNAs (miRNAs), small nucleolar RNAs (snRNAs) long noncoding RNAs (LncRNAs), and a number of other noncoding RNAs with a not yet identified function.

The first noncoding RNA was identified in 1993, and described in two papers published simultaneously [4, 5]. It is now fully established that miRNAs regulate the expression of approximately 30% of known protein-coding genes, and are involved in several biologic processes, including apoptosis, proliferation, differentiation and metastatization [6–8].

These RNA products can regulate gene expression at the post-transcriptional level, functioning as endogenous inducers of the RNA interference (RNAi) pathway, first described by Nobel laureates Fire and Mello [9]. miRNA synthesis begins with the transcription of a longer

precursor, the pri-miRNA, ranging in size from 100 nt to several kilobases [10]; this is, then, processed by Drosha, an RNase III enzyme, that forms a complex with *DGCR8* (DiGeorge syndrome critical region gene; also called Pasha). This complex is known as the "microprocessor complex" and is responsible for the production of the ultimate miRNA precursors (pre-miRNAs) composed by a short hairpin structure of 60–110 nt. Pre-miRNAs are then exported to the cytoplasm by an Exportin-5. In this compartment, the pre-miRNAs are processed by Dicer-1, another RNaseIII enzyme, associated with TRBP/PACT proteins. This process produces a double-stranded miRNA duplex, which is successively unwound by a helicase into a mature miRNA (Figure 1). The product, of approximately 20 nt in length, is then incorporated into the RNA-induced silencing complex (RISC), formed by the miRNA with different isoform of the Argonaute family protein members [11].

The RNAi mechanism to induce gene silencing occurs trough the degradation of the mRNA target. In this case one strand of the RNA duplex (an siRNA in this case), termed the guide strand, is loaded into RISC, where it binds with a perfect pairing to the homologous mRNA target, which is then degraded through the nuclease activity of the Argonaute family of proteins (Figure 2A), in particular Ago2 [12].

Conversely, miRNAs have an imperfect pairing with their target, and even if the same pathway and enzymes are mainly involved, generally this does not bring to the degradation of the target, but to the blocking of the translation, or the storage of the mRNA into the P bodies. In *Drosophila*, the double-stranded miRNAs and siRNAs are loaded into AGO1 and AGO2, respectively. This stringent miRNA/siRNA sorting is due to the intrinsic property, sequence and secondary structure of the small RNA itself [13, 14].

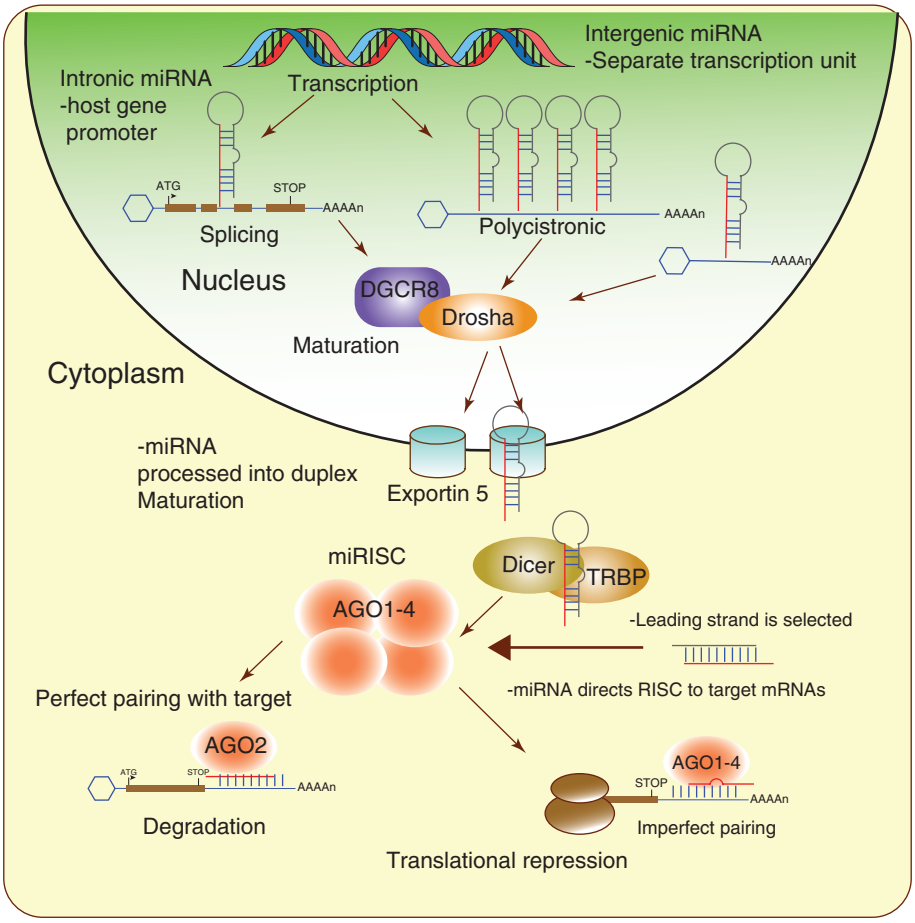

Figure 1: Production and maturation of miRNAs.
The pre-miRNAs are transcribed in the nucleus as shown. After the maturation process exerted by the DGCR8 and Drosha enzymes, they are actively transported from the nucleus to the cytoplasm. In this compartment, the pre-miRNA is processed by Dicer-1, a RNaseIII enzyme, associated with TRBP/PACT proteins. The product is then incorporated into the RNA-induced silencing complex (RISC), composed of the miRNA and different isoform of the Argonaute family protein members (AGO), which then leads to the degradation of target mRNA (AGO2) or translational repression (AGO1-4).

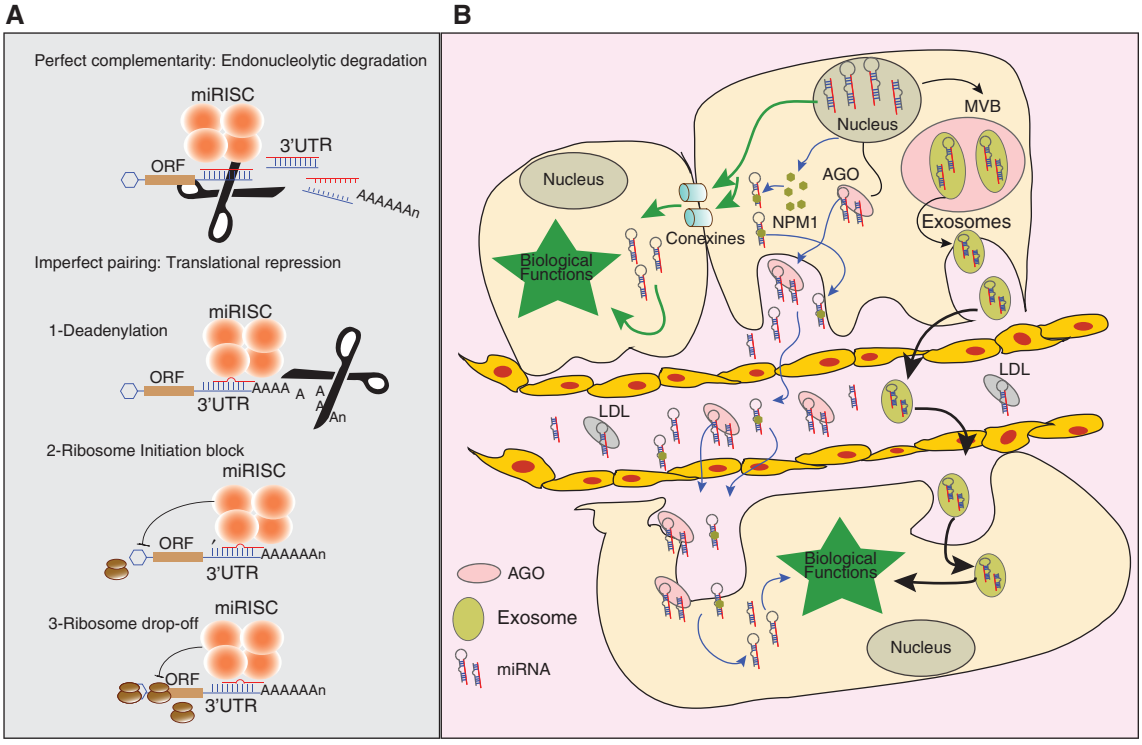

Figure 2: mRNA regulation by miRNAs.
(A) miRNAs typically regulate gene expression by binding to partially complementary target sites in the 3′-UTR of mRNA, reducing its translation. If miRNA and mRNA targets share perfect complementarity, the mRNA will be cleaved and degraded (AGO2 action). Imperfect binding will leads to translational repression, occurring through a variety of different mechanisms: 1) Target mRNAs poly-A can be shorten by deadenylases into P-bodies preventing translation; 2) Prevention of ribosome binding to the 5′ cap, resulting in a block of translation initiation; 3) miRNAs can also block the elongation step of the translation by inducing secondary structure restrictions which lead to a ribosome drop-off. (B) For the cellular release and uptake of C-miRNAs, at least three ways are known, the miRNAs can be transported between cells either mediated by using extracellular vesicles (exosomes or MVBs), by other RNA-binding proteins or direct transfer through cell gap junctions (green arrow flowing). MVBs, multivesicular bodies; NPM1, nucleophosmin 1; AGO2, Argonaute-2; HDL, high-density lipoprotein.

miRNA targeting

Generally, the miRNA-RISC complex binds to 3′ UTRs of target mRNAs by Watson-Crick base pairing (WCP), and imperfect pairing gives rise to inhibition of protein translation (Figure 1A). Target regions for a specific miRNA can also be found in 5′ UTRs and open reading frames, but real targeting in these regions is rare with respect to 3′ UTR [15]. Recently it has also been discovered that they can target other RNA species, including lncRNAs, and other RNAs [16–18].

miRNAs interact with their mRNA target with nucleotide complementarity through their own 5′ ends [15, 19, 20]. Mathematical computational analyses studies showed that the perfect WCPs correspond to the miRNA region between the 2nd and the 7th nucleotide at the 5′ end [20–22]. This region spanning 6 nucleotides (nt) can be considered the “seed”, with further relevance to an additional base pairing at the 8th nt position of miRNA. Furthermore, the presence on the mRNA of an adenine complementary to the 1st nucleotide position of miRNA improves targeting ability of the miRNA.

Based on these findings, four canonical site types (CSTs) have been determined, these include one 6mer, two 7mers and one 8mer. The latter site contains the seed match flanked at position 8 by the A at position 1 [20]. The best 7mer site can be considered the 7mer-m8 site, that shows the presence of the seed, improved by a match to miRNA nucleotide 8 [20–22]. Another 7mer, the 7mer-A1 site, is also functional, it contains the seed match plus an A at target position 1 [20]. The 6mer is the perfect 6-nt match to the miRNA seed (miRNA nucleotides 2–7) [20].

Microarray experiments using ectopic miRNA expression, allowed measuring of their impact on the cell and tissue transcriptome. The results showed that a large number of mRNA targets were directly down-regulated

[23, 24]. Whole proteomic analyses and ribosome profiling showed that miRNAs are able to down-regulate gene expression mainly through mRNA destabilization rather than a classical translational repression by promoter repression [19, 25, 26].

Circulating miRNAs

Mature miRNAs can be detected in blood plasma, serum and also in a wide variety of biological fluids [27]. It is now known that circulating miRNAs (C-miRNAs) can regulate several key cellular processes and target gene expression in recipient cells, thus conditioning cellular development, differentiation, proliferation, cell death and metabolism. Recent evidence indicates that C-miRNAs can regulate target gene expression in very distant recipient cells, thus behaving in a hormone-like way [28]. To elucidate miRNAs regulatory functions, it is important to know the mechanism of their release, packaging and uptake, as recent studies suggest that C-miRNAs could be subjected to selective packaging and release [29].

Several models for the release of C-miRNAs have been proposed (Figure 2B). As is the case for other components of the cell cytoplasm, these molecules can be released from injured cells, chronic inflammation or necrosis [30, 31], with a passive mechanism. This has been demonstrated for some miRNAs like miR-208, miR-122, miR-192, miR-21, miR-200c and miR-423 [32–35], which are probably released from the heart, liver and kidney after injury. Otherwise they can be released by active secretion via membrane vesicles such as exosomes, L-exosome, microvesicles or apoptotic bodies [36]. This is an important process, as it has been demonstrated that the proportion of miRNA is higher in exosomes than in their parent cell [37, 38]. This has been widely confirmed in profiling studies, showing that miRNAs are not simply randomly incorporated into exosomes. In fact, the intracellular miRNA expression levels in a variety of cell lines and their respective derived from exosomes, showed that a subset of miRNAs preferentially enter exosomes [39].

Complexes containing lipoproteins (e.g. high-density lipoprotein [HDL]) seems to also be responsible for active secretion [40]; for example, native HDL can readily associate with exogenous miRNAs and deliver genetic material to recipient cells with functional targeting capabilities [41], producing altered gene expression [40], as well as RNA binding proteins like AGO2 [42] and nucleophosmin 1 [43]. In fact, the RNA-binding protein nucleophosmin plays a key role in exportation, packaging and protection of extracellular miRNAs [43].

For example, almost 90% of the plasma and serum miRNAs are cofractionated with protein complexes rather than encapsulated by vesicles [44].

The uptake by recipient cells of C-miRNAs is essential to produce a proper regulatory function. For this process exosomal proteins like annexins, tetraspanins (CD63, CD81, CD82 and CD9), and heat-shock proteins (Hsp60, Hsp70 and Hsp90) are important [28]. Currently, different pathways for the uptake of C-miRNAs have been proposed (Figure 2B).

The first considers the internalization via endocytosis of the vesicle-enclosed miRNAs, either by phagocytosis or by a direct fusion with recipient cells plasma membranes. Another one considers the recognition of target cells by molecules or specific receptors located on the microvesicle and recipient cell surfaces, thus permitting specific miRNA uptake. Recent studies also showed the importance of cell gap junctions in making the miRNAs transfer possible (Figure 2B) [45–47].

Circulating miRNAs in disease diagnostics

An extraordinary knowledge on miRNAs as endogenous mediators of RNA translation has been obtained in recent years, underlining their potential diagnostic relevance (Figure 3).

C-miRNAs may even be differently secreted in cells associated with specific pathological conditions [48], and therefore may be used as clinical biomarkers. Numerous evidences indicate that the initiation and progression of several pathologies are “highlighted” or may be influenced by disregulation of miRNAs, due to their ability to have an effect on multiple targets [49], as has been widely demonstrated in cancer [50]. In support of this argument, they are present in body fluids, most notably in blood as well as in urine, saliva, breast milk, pleural, peritoneal and cerebrospinal fluids [27].

The possible use of molecules as biomarkers in human specimens depends on their stability in this environment. In body fluids and in blood there are high levels of RNAses that can rapidly degrade exogenously added mRNAs [31, 51]. The mechanisms behind miRNA’s stability can be explained, as previously stated, by the discovery of their presence in exosomes [52, 53], that can be found in various types of body fluids [54, 55]. Inside cells, exosomes are formed through inward budding of endosomal membranes, and therefore give rise to intracellular multivesicular bodies (MVBs) [51]. Once in body

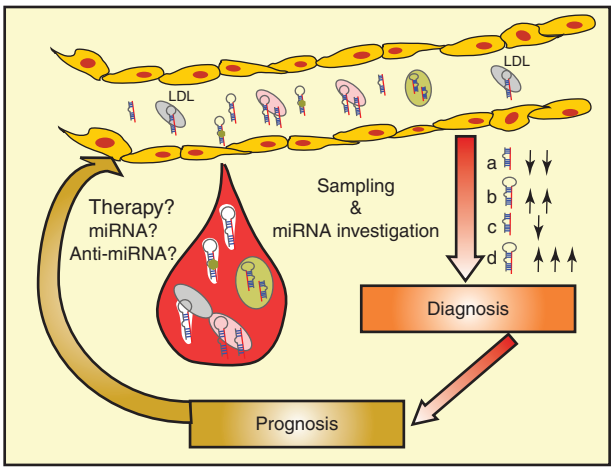

Figure 3: Clinical use of C-miRNAs detection.
C-miRNAs can be easily detected in blood samples, using plasma or serum as the starting material for RNA extraction. The detection of a specific miRNAs signature could give the possibility of an early diagnosis and prognosis, as well a good follow-up evaluation. Importantly the future new therapeutic strategies could be set up using anti-miRNAs or mimic of miRNAs injected directly in blood steam to modify specific cellular processes.

fluids, cell recognition molecules on the microvesicle surfaces or specific receptors on the recipient cell membranes permit specific miRNA uptake. MVBs fuse with the plasma membrane, thus inducing the release of exosomes to the outside of cells [56]. Furthermore, C-miRNAs can transit between cells, in order to perform their functions inside the recipient cells [57]. Extracellular miRNAs can also be transferred from one cell to another to facilitate cross-talk, communication and signal exchange (Figure 2B) [58, 59].

C-miRNAs show almost all the characteristics of a suitable biomarker: their analysis is measurable with non-invasive methods; with modern technologies they can be detected and quantified with a high degree of sensitivity and specificity. miRNAs showed a long half-life in plasma, and their laboratory detection has become rapid and cost-effective, this will allow the early detection of pathological states and easier patient follow-up.

C-miRNAs expression profile has been used to investigate differences between patients and healthy individuals, highlighting their potential use as easily detectable biomarkers [60]. They can also be useful to predict treatment response and recurrence (Figure 3). However, there are examples in the literature in which the data meta-analysis reported a lack of consistency across different profiling studies [61]. This demonstrates that the technical approach needs to be refined to successfully use the C-miRNAs signature in clinical practice.

SNPs in miRNA genes affect their biogenesis and function

Single nucleotide polymorphisms (SNPs) occur in 1% or more among the human population. These SNPs, when present in the coding region can result in non-synonymous changes, resulting in an amino acid change or the introduction of a stop codon, thus leading to diseases. Recently, the analysis of the SNPs identified in non-coding regions showed that about 90% of functional SNPs obtained in genetic GWAS reside in non-coding regions [62]. These SNPs have been called regulatory SNPs or rSNPs because they affect transcriptional regulation or post-transcriptional gene expression [63]. Even taking into account the complexity of miRNA to mRNA pairing, the introduction of a variation or SNP in the recognition sequence, located in the 3′-UTR of a gene, can introduce or remove target sequences or modify the efficiency of miRNA binding. This has been shown to have effect to the expression levels of numerous proteins that have been associated with various disorders [64, 65].

Methodological challenges

Pre-analytical issues

It is well known that in the total testing process, steps occurring before sample analysis, known as the pre-analytical phase, can affect the final results with an estimated error rate of 46%–68% [66]. Pre-analytical variables including biological variation, sample type, sample collection, storage and transportation might be critical confounders in miRNAs profiling [67]. Although studies on miRNAs' pre-analytics are in their infancy with respect to other blood biomarkers, it is reported that patient-related status and sample quality influence miRNAs expression and concentration [68–70]. A number of studies, moreover, demonstrated a correlation between miRNAs concentration and blood cell count [71–73], and this indicates that, to obtain reliable results, venepuncture should be performed according to correct guidelines [74]. In addition to hemolysis and clot formation, also icterus and lipemia influence miRNAs concentration assessed by spectrophotometric methods [75]. Sample type should be carefully evaluated and defined before introducing a miRNAs test in the diagnostic routine in order to obtain comparable data. Plasma and serum samples have been compared in many studies to identify the best matrix for miRNAs profiling, however results are still controversial [30, 31, 76–79].

To date, plasma is considered the sample of choice in studying C-miRNAs, as cellular RNA released into the serum during the spontaneous coagulation process may change the repertoire of C-miRNAs. Higher concentrations of miRNAs were in fact more consistently found in sera than in plasma samples [80]. However, residual platelets are also identified in plasma samples. In this context, type of anticoagulant used and centrifugation protocol to separate plasma fraction from the whole blood, have a great impact on overall miRNAs content. Page et al., identified significant differences due to separation protocol in 72% of the measured miRNAs [81]. Basso et al. proved that, in samples collected in tubes containing EDTA as the anticoagulant, a two steps centrifugation protocol is more suitable than the one-step protocol in removing contaminating platelets. Thus, an additional centrifugation step is highly recommended in these samples [82]. Although the main differences are due to platelets contamination, cell debris and microparticles can also be present in plasma samples. In this case, the removal is allowed by an additional filtration step [83].

Among the other anticoagulants commonly used in clinical practice, lithium-heparin should be avoided because it has been proved to significantly reduce miRNAs concentration [82], interfering with reverse transcriptase [84] and polymerase enzymes used in the PCR reaction [79]. Anyway, miRNAs can be detectable in samples already collected in lithium-heparin tubes if adequately treated with heparinase [75] or LiCl [85]. In this context, it is very important to also know whether the patient is taking certain medications, as miRNAs concentration was significantly reduced in patients taking heparin as an anticoagulant in cardiovascular diagnostics and interventions [78].

Similar to heparin, citrate has also been shown to interfere with qRT-PCR miRNAs assessment [86]. Furthermore, sodium citrate seems to trigger hemolysis enhancing the release of confounding miRNAs and the final concentration of plasma miRNAs up to 50-fold [87]. EDTA-plasma yielded the best miRNAs profiles compared to citrate and heparin-plasma [88]. The comparison of four different anticoagulants, EDTA, heparin, sodium citrate and sodium fluoride/potassium oxalate (NaF/KOx), demonstrated that the most reproducible results in miRNAs quantification is provided by using tubes containing NaF/KOx [79].

miRNAs quantification might also be influenced by plasma volume, where using a high plasma volume to isolate miRNAs allows obtaining a sufficient yield of miRNAs, but also an excess of polymerase inhibitors (hemoglobin, lactoferrin and IgG) that might limit the accuracy of the analysis. On the other hand, in a low sample volume, there is a small amount of endogenous inhibitors but the amount of miRNAs is too low to be profiled [79].

Storage temperature was identified as a relevant factor affecting miRNAs expression profiles in serum. In particular, room temperature seems to promote cell degradation, cytokine's release and variations in miRNAs profile in serum sample. By contrast, miRNAs levels were stable at room temperature for at least 24 h in whole blood samples [82]. Serum miRNAs were instead proved to be stable for up to 72 h when refrigerated at 4 °C or frozen at –20 °C, because of the reduction of RNase activity at low temperature [89]. miRNAs stability after long-term storage showed that the total amount of miRNAs in serum was rather stable for 2–4 years at –20 °C but was significantly decreased after 6 years of storing and even more after 10 years. Moreover, repeated freeze-thaw cycles resulted in a significant decrease in miRNAs concentration when compared to continuous storage at –80 °C [90].

Analytical issues

Another limiting factor for the identification of reliable C-miRNA signatures could be represented by the methods used for nucleic acid extraction and amplification.

To date, several manufacturers developed miRNAs extraction methods. Those used most commonly and for a while are based on the phenol:chloroform technique, while newer methods, allowing faster and partially-automated extraction, provide for the adsorption of RNA on a silica mini-column or beads or glass fiber filters. Today, the efforts conducted to optimize current methods allow the analysis of miRNAs signature by a starting volume lower that 1 mL of whole blood. miRNAs profile analysis can still require more initial volume in the case of children, the elderly or oncological patients [91]. Moreover, the RNA quantity can dictate the method used for subsequent miRNAs analyses. It has also to be kept in mind that the commonly used purification methods (such as the miRNeasy® kit-Qiagen, Hilden, Germany, or the miRVana™ PARIS™ kit-Applied Biosystems/Thermo Fisher, Waltham, MA, USA) have been designed to isolate total RNA, which also includes miRNAs and other small RNAs. As miRNAs are molecules physically and chemically different from the larger RNA, the quality and composition of RNA extracted have been demonstrated to be very different depending on the method used. The matter is further complicated by the fact that C-miRNAs, to escape RNases degradation, are associated with proteins (e.g. Argonaute) and lipoproteins or transported within exosomes. In this context, methods used to extract miRNAs from cells or tissues not may be

suitable for extracting C-miRNAs under any situations, probably not even for miRNAs that are exosome-enclosed, as exosomal membranes have a different lipid composition from cellular membranes [92].

Eldh et al., by comparison of seven different methods for exosomal RNA extraction, demonstrated an extreme variation in RNA yield and signature among different methods. In particular, the extraction of exosomal RNA using phenol (Trizol) pure or combined to column-based technique (miRCURY™ RNA Isolation Kit, Exiqon-QIAGEN, Vedbaek, Denmark) were shown to be the most efficient methods in terms of yield and specificity (small RNAs rather than total RNAs) [93]. However, while Trizol is widely employed, its use should be carefully evaluated in this setting considering findings that report the selective Trizol-dependent loss of pre-miRNAs, small interfering RNAs and transfer RNAs molecules extracted from cells [94].

On the basis of these considerations, we can conclude that the selection of the miRNA extraction kit should be based on several factors: available starting volume, miRNAs species cellular or circulating, and method of subsequent miRNAs expression analysis.

The method used to evaluate quality and quantity of extracted miRNAs, can also influence the expression analysis. Although the spectrophotometric method (e.g. Infinite 200 PRO Nanoquant and Nanodrop 2000) is the most common one to determine concentration and contamination by proteins or phenols, it showed low specificity and sensitivity. It, in fact, does not discriminate miRNAs from total RNAs and other small RNAs, and often fails in revealing phenol contamination as the absorbance of phenol (270 nm) is very close to that of nucleic acids (260 nm) [95]. Moreover, because the extracellular miRNAs are less than 1% of the total RNA recovered, their concentration is often under the detection limits of spectrophotometric techniques. To avoid this issue, novel methods have been proposed in this context. Garcia-Elias et al., by comparison of six methods used to quantify miRNAs, demonstrated that both spectrophotometers, Infinite® 200 PRO Nanoquant and Nanodrop 2000, detected contaminants, proteins and other RNA molecules in addition to miRNAs, thus determining an overestimation of the studied target; on the contrary Qubit 2.0 Fluorometer provided the most accurate quantification of miRNA content, although RNA-seq confirmed that only ~58% of small RNAs in plasma are true miRNAs. Finally, the Agilent 2100 Bioanalyzer Pico Chip and Small Chip kits did not give a reliable quantification method for plasma samples but provided valuable information on RNA profiles [96]. The latter system, in fact, examines the presence of small RNAs between 6 and 150 nucleotides and determines their quality assigning an RNA integrity number (RIN) by using an algorithm based on ribosomal RNA detection [97, 98]. It has been demonstrated that a RIN > 6 is acceptable to profile a large number of miRNAs from both plasma and serum samples, by contrast RIN < 6 might indicate the presence of degraded miRNAs [99, 100]. However, in this case, individual miRNAs could be analyzed by reverse transcriptase quantitative real-time polymerase chain reaction (RT-qPCR). Finally, the Small Chip kit is not applicable when miRNAs are isolated from exosomes, being that ribosomal RNAs are not reliable in exosomes.

However, considering the unique chemical properties of miRNAs, the choice of platform used to determine miRNome or detect and quantify specific miRNA-biomarker candidates represents the other main analytical variable. Firstly, miRNAs represent a very small part (approximately 0.01%) of the total RNA content, so that they must be first selectively detected from other RNA species present in the sample [87]. Furthermore, the short length of mature miRNAs and lack of a poly(A) tail pose challenges for annealing to traditional primers designed for reverse transcription and PCR [87, 91]. In addition, the high variance in miRNAs GC content is reflected in a wide variance in melting temperature, thus complicating the standardization of the annealing reaction when hundreds of miRNAs are analyzed in parallel. Finally, the high sequence conservation among miRNAs family members (usually differing for a single nucleotide), and the presence of "isomiRs" which present variations with respect to the reference miRNA sequence, make it difficult to discriminate individual miRNA from one another [87]. Despite these issues, techniques to be used to profile miRNAs are numerous and include RT-qPCR, digital PCR (ddPCR), hybridization-based techniques (e.g. microarrays, NanoString), and next generation sequence (NGS). To date, however, there is still a lack of consensus regarding optimal methodologies or technologies for miRNAs detection in liquid biopsy. Furthermore, the individual miRNA expression among different profiling platforms is not comparable. Moldovan et al., extensively reviewed methodological aspects, advantages and limitations of the most common methods used [91].

Briefly, a large part of these, especially microfluidics-based approaches, is primarily applicable to tissues and cell culture-derived miRNAs because of the large volume requested as starting material. Conversely, RT-qPCR is considered the gold standard for gene expression measurements, displaying better sensitivity than array technologies. PCR plates, in fact, can accommodate a larger RNA input volume than the previously mentioned approaches, thus representing a suitable technique in the case of samples containing low concentrations of miRNAs (e.g. plasma, serum

and other biofluids). The most accepted method for miRNAs quantification by using RT-qPCR is a two-step approach that first includes the use of looped miRNA-specific reverse transcription primers and then the use of specific probes.

More recently ddPCR has been proposed as the promising approach in the absolute quantification of miRNAs, because external references are not required, and it reveals greater precision and improved day-to-day reproducibility when compared to RT-qPCR.

However, the use of RT-qPCR or ddPCR is limited in number of detectable miRNAs. In this context microarray platforms are capable of analyzing a large number of miRNAs in parallel, they are suitable for comparison of relative abundance of specific miRNAs between two conditions (e.g. disease vs. healthy). However, the obtained data are typically validated by a second method, usually RT-qPCR [91].

Overall, NGS platforms are considered the most promising technology for miRNAs' expression profiling, and in particular for miRNAs discovery, as they are able not only to detect known miRNAs, but also new miRNAs and to distinguish miRNAs differing for a single nucleotide as well as isomiRs. Furthermore, compared to microarrays, NGS is extremely sensitive as it is able to detect one miRNA copy per cell, and to allow relative quantification of miRNAs that are estimated to vary in abundance by four orders of magnitude. It should be noted that NGS also presents disadvantages, that should be considered before introducing NGS into a laboratory routine [87].

Decisively, the results validated using the RT-qPCR could be considered as the only way to confirm data and compare different studies [101].

The accuracy of validation phase, however, critically depends on proper normalization of the data. Inappropriate normalization of qRT-PCR data can, in fact, lead to incorrect conclusions. The normalization is necessary to minimize as much as possible variation between groups, e.g. "healthy" vs. "disease". Although an ideal normalizer should be expressed equally across all samples and along with the target in the sample of interest, it still does not exist [102], and for C-miRNAs profiling, it remains a challenge. To date, several small RNA species [103–111], have been described as endogenous controls. U6 and miR-16 are the most commonly used in miRNAs studies. However, it was recently suggested that U6 is an unsuitable normalizer for the quantification of miRNAs in some specimens [112–115]. Also, miR-16, has been demonstrated to be an unsuitable endogenous control in serum samples. Despite these considerations, such normalizers have been specifically identified for certain diseases. In particular, miR-16 and miR-93 for gastric cancer [116], SNORD43 for urological malignancies [117], a combination of miR-191-5p and RNU6 for colorectal cancer [118] or RNU91 for pancreatic ductal adenocarcinoma [119].

Clinical miRNA diagnostics

C-miRNAs may be of value in establishing the stage of several pathologies like cardiovascular, vessels, muscular, neurological and metabolic diseases, cancer as well as in monitoring physiological changes, such as those related to physical exercise.

Different studies [120–123] have shown that C-miRNA's levels change depending on the stage of disease and it is clear that the down/up-regulation or the deregulation could be used as biomarkers in clinical diagnostics, for early detection, prognosis and therapeutic guidance.

Here, we report (Tables 1–4) the numerous data collected in recent years of the involvement of miRNAs in several disease and in cancer [120–123].

Circulating miRNAs in cardiovascular diseases

In recent years, the investigations of the presence and the role of miRNAs in cardiovascular biology and disease have been dramatically expanded. Today the value of post-transcriptional regulation, mediated by miRNAs in cardiovascular homeostasis, in heart disease pathogenesis, diagnosis, and prognosis are well established in the scientific community [124, 125] (Table 1).

Also, the field of vascular pathophysiology has seen an explosion of results on miRNAs, with the recognition of their role in controlling smooth muscle cell proliferation and maturation, vasculogenesis, neoangiogenesis or endothelial function.

The fact that a number of these miRNAs can be found in the circulatory torrent *in vivo* in normal and pathologic conditions is fundamental. For this reason, they represent a good target to analyze the cardiologic and vascular state of patients [126] and for therapeutic efficiency prediction and follow-up.

Acute myocardial infarction (AMI)

In AMI patients, a number of miRNAs have been found deregulated with respect to healthy patients (Table 1). Among the identified miRNAs, three different groups can be distinguished. The first one includes miR-1, 30c, 133a,

Table 1: C-miRNAs correlated to diseases of the heart district.

District	Pathology	Regulation	miRNA	References
Heart				
	Acute myocardial infarction	Up-regulated	1, 30-c, 133-a, 134, 145, 186, 208-a, 208-b, 499	Kondkar et al. 2015; Bush et al. 2016; Wang et al. 2014; Meder et al. 2011; Adachi et al. 2010
		Down-regulated	663-b, 1291	Bush et al. 2016; Kondkar et al. 2015
		Modified in specific conditions	19-a, 126, 150, 223, 320-a/b, 380	Bush et al. 2016
	Heart failure	Up-regulated	21, 27-a, 29-a, 155, 210, 499, 660-3p, 665, 1285-3p, 4491	Bush et al. 2016; Corsten et al. 2010; Li et al. 2016
		Down-regulated	1, 142, 145, 150	Bush et al. 2016; Zhang et al. 2017; Devaux et al. 2013
		Modified in specific conditions	133-a/b, 146-a, 423	Bush et al. 2016
	Stroke	Up-regulated	21, 151-a	Sørensen et al. 2014; Bush et al. 2016
		Down-regulated	126	Bush et al. 2016
		Modified in specific conditions	16, 30-a, 106-b, 320-d	Bush et al. 2016
	Acute coronary syndrome	Up-regulated	1, 133-a/b, 208-b	Kondkar et al. 2015
	Coronary artery disease	Up-regulated	21, 133-a/b, 199-a	Bush et al. 2016
		Down-regulated	1, 17, 92-a, 126, 145, 155	Wang et al. 2014; [88]
	Bicuspid aortic valve	Up-regulated	130-a	[131]
		Down-regulated	122, 718, 486	[130, 131]

Name and year references details are provided in Supplementary Material.

Table 2: C-miRNAs detected in vessel and muscular modifications.

District	Pathology	Regulation	miRNA	References
Vessels				
	Aortic aneurysm	Down-regulated	15-a, 21, 29,-a, 103, 124-a, 143, 145, 155	Bush et al. 2016
Muscle				
	Duchenne muscular dystrophy	Up-regulated	1, 20, 31, 133-a/b, 206, 208-a/b, 486, 499	Cacchiarelli et al. 2011; Zaharieva et al. 2013; Ma et al. 2015; Li et al. 2014; Alexander et al. 2014
	Myotonic dystrophy	Up-regulated	1, 29-b, 133-a/b, 206	Koutsoulidou et al. 2017; Ambrose et al. 2017
	Inflammatory myopathies	Up-regulated	21	Shimada et al. 2013
	Fibromyalgia syndrome	Up-regulated	320-a	Bjersing et al. 2014
		Down-regulated	30-b-5p, 103-a-3p, 107, 142-3p, 151-a-5p, 374-b-5p, let-7-a-5p	Bjersing et al. 2014
	ALS	Up-regulated	143-3p, 206	Waller et al. 2017; Ma et al. 2015
		Down-regulated	374-b-5p	Waller et al. 2017
	Exercise	Modified in specific conditions	1, 133-a/b, 206, 208-a/b, 486, 499	Kirby et al. 2013
	Aerobic exercise	Up-regulated	20-a	Baggish et al. 2011
		Down-regulated	486	Aoi et al. 2013
	Acute resistance exercise	Up-regulated	149	Sawada et al. 2013
	High fitness Level	Down-regulated	21, 210, 222	Bye et al. 2013

Name and year references details are provided in Supplementary Material.

134, 145, 186, 208 a, 208b and 499, of which significantly high levels have been found. The second group is represented by miR-663b and miR-1291, that present low levels of expression with respect to normal (Refs in Table 1). A third group of miRNAs, like miR-19a, 126, 150, 223, 320a, 320b and 380 have also been found to be disregulated (Refs in Table 1). The first group, when standardized, could be used for the early diagnosis of AMI, whether

Table 3: C-miRNAs correlated to metabolic diseases.

Metabolic disease	Pathology	Regulation	miRNA	References
	Type 1 diabetes	Up-regulated	24, 25, 26-a, 27-a/b, 29-a, 30-a-5p, 148-a, 152, 181-a-5p, 200-a, 210	Chevillet et al. 2014; Nielsen et al. 2012
	Type 2 diabetes	Up-regulated	9, 24, 28-3p, 29-a, 30-d, 34-a, 124-a, 144, 146-a, 375	Kong et al. 2011; Wang et al. 2014; Zampetaki et al. 2010
		Down-regulated	15-a, 20-b, 21, 24, 29-b, 126, 150, 191, 197, 223, 320, 486	Kong et al. 2011; Wang et al. 2014; Zampetaki et al. 2010

Name and year references details are provided in Supplementary Material.

circulating miR-1 and 29-b described as adverse ventricular remodeling after acute myocardial infarction, could be used in follow-up [127].

Heart failure and acute coronary syndrome

In patients with heart failure the miR-21, 27a, 29a, 155, 210, 499, 660-3p, 665, 1285-3p and miR-4491 levels were found to be higher than in healthy controls, while the miR-1, 142, 145 and miR-150 levels were low. miRNA levels of 133a, 133b, 146a and 423 were found to be disregulated in a number of patients. Circulating miRNA-22-3p was previously linked to this pathology and tested for its temporal expression level as a predictive factor of prognosis in a prospective cohort of chronic heart failure patients [128]. Interestingly, in acute coronary syndrome the levels of circulating miR-1, 133a, 133b and 208b were found to be upregulated (Refs in Table 1), and another study has identified eight circulating miRNAs, that can be used as prognostic biomarkers for this pathology [129].

Coronary artery disease (CAD)

In patients with CAD, recent studies found that miR-21, 133a, 133b and miR-199a levels were higher with respect to normal controls while the expression of miR-1, 17, 92a, 126, 145 and 155 were downregulated (Table 1). However, further extensive overviews of expression levels of C-miRNAs in CAD and non-CAD patients demonstrated variations in different studies, due mainly to the different parameters used. Further trials should be conducted to define the role of miRNAs as diagnostic markers in CAD [130].

Bicuspid aortic valve (BAV)

BAV is a frequent congenital cardiac malformation. It is often associated with a dysfunction of the aortic valve, with the progressive dilation of the ascending aorta, this dilation generally leads to aortic regurgitation. The levels of miR-130a were up-regulated while levels of miR-122, 486 and miR718 were found to be down-regulated. This C-miRNAs signature integrates these markers in the complex association of the bicuspid morphology of the aortic and the progressive dilation of the ascending aorta [131] and Refs in Table 1.

Circulating miRNAs in vessels pathology

The proper development of the vascular system is critical in embryo development and survival. Two main processes, the vasculogenesis and angiogenesis give rise to blood vessel formation during development. The extension of new segments from pre-existing vessels is driven by the vascular endothelial growth factor (VEGF) and hypoxia. Currently a number of miRNAs have been identified to be involved in blood vessel development, as well in pathological conditions [132, 133], thus giving the possibility to analyze their expression modifications in blood flux (Table 2).

Aortic aneurism (AA)

In a large cohort of patients, a number of miRNAs has been identified with altered expression of C-miRNAs in AA patients when compared to controls. Assessment of miRNAs expression may offer an opportunity to predict disease progression and aneurysm growth. In fact, in patients with AA, miR-103, 15a, 21, 29a, 124a, 143, 145 and miR-155′ levels in plasma were modified with respect to healthy controls [134] and referenced in Table 2.

Stroke

The etiology of ischemic stroke is mediated by a complex cascade of molecular events, demonstrated to be in part

Table 4: C-miRNAs in cancer.

District	Cancer type	Regulation	miRNA	References
Blood	CLL	Up-regulated	20-a, 150, 195, 638	Moussay et al. 2011; [120, 154]; Tanaka et al. 2009
		Down-regulated	15-a, 16, 29, 34-a, 155, 181-b	Yeh et al. 2016
	AML	Up-regulated	10-a-5p, 93-5p, 129-5p, 155-5p, 181b-5p, 320-d, 335	Zhi et al. 2013; [120]; Lin et al. 2015
	Diffuse large B-cell lymphoma	Up-regulated	15-a, 16, 21, 155, 210	Fang et al. 2012; Lawrie et al. 2008
	Large B-cell lymphoma	Up-regulated	21, 29-c, 34-a, 155, 210	[120]
Gastrointestinal tract	Colorectal cancer	Up-regulated	17-3p, 17-92a, 18-a, 19-a, 19a-3p, 20-a, 21, 21-a, 23-a, 27-b, 29-a, 92, 92-a, 92a-3p, 106-a, 125-b, 130-b, 133-a, 141, 143, 145, 148-a, 181-b, 183, 193a-3p, 221, 223-3p, 326, 338-5p, 342-3p, 372, 422-a, 484, 532-3p, 885-5p, let7-a	Ng et al. 2009; Matsumura et al. 2015; Luo et al. 2013; Zheng et al. 2014; [80]; Yong et al. 2013; Kjersem et al. 2014; Yamada et al. 2015; Huang et al. 2010; Li et al. 2015; Cheng et al. 2011; Ramzy et al. 2015; Yuan et al. 2015; Pu et al. 2010; Fang et al. 2015; Yu et al. 2016; Hur et al. 2015; Ogata-Kawata et al. 2014
		Down-regulated	10-b, 24, 29-b, 30-b/c/d, 34-a, 146-a, 194, 320-a, 423-5p	Heneghan et al. 2010; Fang et al. 2015; Basati et al. 2016; Ho et al. 2015; Nugent et al. 2012
		Modified in specific conditions	126, 155	Yin et al. 2014; Hansen et al. 2015; Heneghan et al. 2010; Lv et al. 2015
	Gastric cancer	Up-regulated	1, 16, 17, 17-5p, 18, 20, 21, 25, 27-a, 34-a, 92-a, 106-a/b, 146-a, 148-a, 192, 196-a, 199a-3p, 200-c, 378, 421, 451, 486-5p, let7-a	Liu et al. 2011; Zhu et al. 2014; Tsujiura et al. 2010; Zhou et al. 2012; Cortez et al. 2009; Kim et al. 2013; Chen et al. 2014; Tsai et al. 2012; [170]; Valladares-Ayerbes et al. 2012; Liu et al. 2012
		Down-regulated	122, 195-5p, 375	Chen et al. 2014; Gorur et al. 2013; Zhang et al. 2012
	Hepatocell carcinoma	Up-regulated	1, 15-b, 18, 25, 101, 122, 130-b, 206, 221, 222, 223, 375, 500, 618 (urine), 885-5p, let7-f	Li et al. 2010; Liu et al. 2012; Li et al. 2012; Fu et al. 2013; Xu et al. 2011; Li et al. 2011; Qi et al. 2011; Yamamoto et al. 2009; Abdalla et al. 2012; Gui et al. 2011
		Down-regulated	16, 199-a, 650 (urine)	Qu et al. 2011; Abdalla et al. 2012
		Modified in specific conditions	21, 92-a	Xu et al. 2011; Tomimaru et al. 2012; Qi et al. 2011; Shigoka et al. 2010; Li et al. 2010
	Pancreatic cancer	Up-regulated	21, 155, 196-a, 210, 155, 200-a/b	Wang et al. 2009; Kong et al. 2011; Miller et al. 2008; Li et al. 2010
Breast	Breast cancer	Up-regulated	1, 10-b, 10b-5p, 16, 18-b, 21, 27-a, 34-a, 92-a, 101, 103, 106-a, 107, 125-b, 127-3p, 130-a, 132, 133-a/b, 146-a, 148-b, 181a-5p, 182, 191, 195, 199°-5p, 202, 210, 373, 382, 409-3p, 425, 451, 484, 589, 652, 801, let7-b/g	Ng et al. 2009; Matsumura et al. 2015; Luo et al. 2013; Zheng et al. 2014; [80]; Yong et al. 2013; Kjersem et al. 2014; Yamada et al. 2015; Huang et al. 2010; Li et al. 2015; Cheng et al. 2011; Ramzy et al. 2015; Yuan et al. 2015; Pu et al. 2010; Fang et al. 2015; Yu et al. 2016; Hur et al. 2015; Ogata Kawata et al. 2014; Chan et al. 2013; Chen et al. 2013; Mangolini et al. 2015; Stückrath et al. 2015; Cookson et al. 2012; Asaga et al. 2011; Roth et al. 2010; Eichelser et al. 2014; Kleivi Sahlberg et al. 2015; Wang et al. 2010; Mar-Aguilar et al. 2013; Cuk et al. 2013; Ferracin et al. 2015; Wang et al. 2013; Heneghan et al. 2010; Shin et al. 2015; Schrauder et al. 2012; Müller et al. 2014; Ng et al. 2013; Zearo et al. 2014; Zhao et al. 2010
		Down-regulated	30-a,126, 148b-3p, 199-a, 205, 335, 652-3p, 718, let7-c/d	Zeng et al. 2013; Mangolini et al. 2015; Wang et al. 2010; Zhang et al. 2015; Schrauder et al. 2012; Zhao et al. 2010

Table 4 (continued)

District	Cancer type	Regulation	miRNA	References
		Modified in specific conditions	145, 155, 215, 299-5p, 376 a/c, 411, 452	Mar-Aguilar et al. 2013; Roth et al. 2010; Zhu et al. 2009; Sun et al. 2012; Van Schooneveld et al. 2012; Cuk et al. 2013
Ovary	Epithelial ovarian cancer	Up-regulated	21, 29-a, 92-a, 93, 99-b, 126	Resnick et al. 2009
		Down-regulated	127, 155	Resnick et al. 2009
Prostate	Prostate cancer	Up-regulated	16, 18-a, 20-a, 21, 24, 26-b, 30-c, 34-b, 92-a/b, 93, 100, 106-a, 107, 125-b (ejaculate), 130-b, 141, 146-a, 183 (urine sediment), 197, 200-a/b/c, 200-c (ejaculate), 221, 222, 223, 328, 429, 451, 485-3p, 486-3p, 574, 574-3p, 636, 640, 766, 874, 885-5p, 889 (urine supernatant), 1290, hsv1-H18 (urine), hsv2-h9-5p (urine), let7-a/c	Lodes et al. 2009; Morimura et al. 2011; Lin et al. 2014; Kotb et al. 2014; Li et al. 2015; Kachakova et al. 2015; Mihelich et al. 2015; Bryant et al. 2012; Roberts et al. 2015; Westermann et al. 2014; Stephan et al. 2015; Singh et al. 2014; Lewis et al. 2014; Huang et al. 2015; Yun et al. 2015
		Down-regulated	155, 205 (urine), 205 (urine sediment), 214 (urine), 409-3p	Heneghan et al. 2010; Srivastava et al. 2013; Stephan et al. 2015; Nguyen et al. 2013; Mihelich et al. 2015; Li et al. 2015
		Modified in specific conditions	103, 375	Kachakova et al. 2015; Lodes et al. 2009; Huang et al. 2015
Lung	Lung cancer (NSCLC)	Up-regulated	10-b, 21, 24, 25, 29-a, 29-c, 30-d, 32, 34, 133-b, 140-5p, 141, 142-5p, 148-a, 182, 193-b, 197, 200-b, 210, 223, 301, 432, 486, 566, 574-5p, 652, 660, 1254	Shen et al. 2013; Franchina et al. 2014; [30]; Bianchi et al. 2011; Zhu et al. 2014; Hu et al. 2010; Zhou et al. 2015; Rodriguez et al. 2014; Zheng et al. 2011; Nadal et al. 2013; Kim et al. 2015; Foss et al. 2011; Boeri et al. 2011
		Down-regulated	1, 17, 19-a/b, 20-a, 20-b, 22 (pleural effusion), 26-a/b, 27-a, 28-5p, 30-b/c, 30e-3p, 92-a, 98, 103, 106-a, 139-5p, 142-3p, 146-b, 148-b, 185 (pleural effusion), 191, 198 (pleural effusion), 204, 221, 328, 331-3p, 342-3p, 361-3p, 374-a, 376-a, 429, 484, 499, 625, let7-a/b/d/f	Hu et al. 2011; Bianchi et al. 2011; Aushev et al. 2013; Silva et al. 2011; Shin et al. 2014; Heegaard et al. 2012; Boeri et al. 2011; Han et al. 2013; Guo et al. 2015; Zhu et al. 2014; Hu et al. 2010
		Modified in specific conditions	21 (sputum and BAL), 22, 143 (sputum and BAL), 155 (sputum), 205, 210 (sputum and BAL), 372 (sputum and BAL), 486-5p	Kim et al. 2015; Bianchi et al. 2011; Franchina et al. 2014; Aushev et al. 2013; Boeri et al. 2011

Name and year references details are provided in Supplementary Material.

modulated by posttranscriptional activity. A number of studies have been published, demonstrating the role of miRNAs as possible mediators in pathology of ischemic stroke, via their posttranscriptional gene silencing [135]. Specifically, it has been demonstrated that in the serum and plasma of stroke patients the miR-21 and miR-151a levels were high while the level of miR-126 was low. The levels of miR-16, 30a, 106b and miR-320d were found to be disregulated (Table 2).

Circulating miRNAs in muscular diseases

Duchenne muscular dystrophy (DMD)

In order to replace muscle biopsy in patients affected by DMD non-invasive biomarkers with diagnostic value and prognostic applications have been investigated for a long time. Recently it was shown that the serum levels of some muscle-specific miRNAs were modified in DMD, specifically miR-1, 20, 31, 133a, 133b, 206, 208a, 208b, 486 and miR-499 levels were found to be up-regulated (Table 2).

Myotonic dystrophy 1 (DM1)

DM1 is the most prevalent muscular dystrophy in adults; however, there are very few biomarkers in fluids, mainly blood and derivatives that can give important clinical information of the patient's status. Now miRNAs measured in plasma or serum are acquiring a great potential. Recently the levels of miR-1, 29b, 133a, 133b and miR-206 were found to be up-regulated [136]. Moreover, the levels of miR-21 were found to be high in inflammatory myopathies (Table 2).

Fibromyalgia syndrome (FM)

FM syndrome is characterized by chronic widespread pain. The disease affects about 2% of the general population, for the most part it is found in females. Despite research efforts, the pathogenesis of FM is still not clear. Even though genetic and environmental factors were first considered, recent studies involve neurotransmitters, hormones, cytokines and immune factors. Studies on the miRNAs' concentration in the serum of FM patients demonstrated a possible association with some miRNAs. It has been shown that in patients with FM the levels of miR-320a are up-regulated while the miR-30b-5p, 103a-3p, 107, 142-3p, 151a-5p, 374b-5p and let-7a-5p are down-regulated (Table 2).

Amyotrophic lateral sclerosis (ALS)

ALS is a condition characterized by a selective loss of upper and lower motor neurons. This generates several muscle malfunctions, like weakness, spasticity and atrophy. The ultimate symptom is paralysis, with consequent death due to the failure of respiratory function within 2 to 5 years of symptoms' onset. The mean time necessary from presentation to diagnosis is about 1 year. For this reason, new biomarkers are needed to ameliorate ALS diagnosis and prognosis, and moreover to act as indicators of therapeutic response. The majority of ALS cases are sporadic (sALS) with no clear genetic linkage; the etiology of which remains unknown. Studies in humans have revealed the potential importance of miRNAs in ALS [137]. In the serum of patients with ALS the miR-143-3p, 106 and miR-206 were found to be higher than the healthy controls [138] while the miR-374b-5p was found to be lower (Table 2).

Circulating miRNAs in physical exercise

During physical exercise, the analysis of C-miRNAs demonstrated a variation of their concentration in plasma and in serum. This variation can be due to two main phenomena: the first is that during an intense physical exercise the striate muscles can be subjected to micro-ruptures, thus circulating the myo-miRNAs present in the blood into the muscular cells; the second, more interesting can be due to a signal pathway of muscular cells that use miRNAs as adaptive signalling. During physical exercise a number of miRNAs have been found to be disregulated (Table 2), like miR-1, 133a, 133b, 206, 208 a, 208b, 486 and miR-499, furthermore in aerobic exercise the levels of miR-20a was found to be up-regulated and miR-486 were found to be down-regulated, instead. During acute resistance exercise miR-149 was found to be higher than the steady state and in high fitness level circulating miR-21, 210 and miR-222 were found to be up-regulated (Table 2).

Circulating miRNAs in metabolic diseases

The whole world incidence of diabetes and other metabolic disorders is increasing, with a high number of cases without early diagnosis. This is a problem for

clinicians that have the responsibility of managing the levels of glucose or lipids to avoid other complications. The current methods of diagnosis of metabolic disorders often do not provide clear indications about the etiology of the disease or its evolution and consequent complications. The C-miRNAs found in the blood have been demonstrated to change with physiological conditions and can help to identify the risk of developing metabolic diseases, diabetes or other diseases.

In the patients with type 1 diabetes 12 C-miRNAs were found to be up-regulated (Table 3), whereas, in the patients with type 2 diabetes the levels of C-miR-9, 24, 28-3p, 29a, 30d, 34a, 124a, 144, 146a and miR-375 were found to be higher than in healthy controls. On the other hand, the levels of miR-15a, 20b, 21, 24, 29b, 126, 150, 191, 197, 223, 320 and miR-486 were found to be down-regulated (Table 3).

Circulating miRNAs in neurological diseases

Alzheimer's disease (AD)

At present, approximately 30 million people are affected by dementia worldwide, of which around 60% is due to AD. Considering this incidence, there will be approximately 66 million cases in 2030 and 120 million in 2050 [139]. Pathological changes in AD brains are known to begin decades before the onset of clinical symptoms [140]. From studies on miRNAs levels in the brains of AD patients, and from analysis of miRNAs important in brain biology [141], a number of them have been analyzed in the plasma and serum of patients [142]. Also, in distinguishing between early AD stages in patients with mild cognitive impairment (MCI-AD) from non-demented patients, a panel of six circulating miRNA have been described as promising biomarker candidates [143]. In patients the levels of C-miR-15a and 34c were found to be up-regulated [144, 145]; levels of C-miR-9, 15b-5p, 29a, 29b, 137, 142-3p, 181c, 191-5p, 301a-3p, 545-3p, let-7d-5p and let-7g-5p were found to be down-regulated [146, 147]. However, for miRNAs, to be of clinical relevance as non-invasive biomarkers for AD, a considerable amount of further work is required.

Parkinson's disease (PD)

PD is the second most common neurodegenerative disease. Again, one of the major challenges in the diagnosis of this neurological disorder is the necessity of biomarkers for early detection. Also, in this case the presence of several blood-based miRNAs has been reported to be used as biomarkers.

In patients with PD, miR-16, 26a-2, 30a, 222, 331-5p, 505 and miR-626 levels were found to be up-regulated [148–150]; levels of circulating miR-1, 22 and 29a were found to be low [148].

Circulating miRNAs in cancer

The diagnosis of cancer is presently based on invasive processes and on serological cancer biomarkers (employed in following-up the cancer patients) that have low specificity and sensitivity, especially if they are used in screening of tumors.

Based on these considerations, an ideal cancer biomarker should be highly specific and sensitive, assayed with non-invasive procedures, possibly less expensive and therefore also a candidate for screening diagnosis.

It is for this purpose that C-miRNAs might represent a viable alternative in cancer diagnosis and prognosis and might provide information on chemoresistence and risk of relapses during the follow-up.

In fact, miRNAs levels are altered in cancer because tumor cells have been shown to release miRNAs in circulation [151–153]. For this reason, miRNAs can be detected in biological fluids of cancer patients and, thanks to their stability in body fluids, they could help in comparing healthy individuals and cancer patients during the stages of initiation and progression.

Hematological oncology

Hematological oncology is characterized by a group of tumors that involve bone marrow cells, and the lymphatic and immune systems.

These pathologies derive from the uncontrolled proliferation of circulating blood cells or their myeloid or lymphoid precursors and the release of these cells in the circulation: that is exactly why miRNAs can be found in the serum and plasma of hematological cancer patients.

Here, we report the data related to chronic lympocytic leukemia (CLL), acute myelogenous leukemia (AML) and diffuse large B cell lymphoma.

Leukemia is one of different types of hematological cancer and is characterized by the invasion of malignant myeloid or lymphoid precursors in the circulation.

In patients with CLL, the malignant precursor derives from the lymphoid lineage. In this pathology, a group of miRNAs represented by miR-20a, 150, 195 and miR-638, were found to be up-regulated (Table 4) while the levels of circulating miR-15a, 16, 29, 34a, 155, 181b were found to be low (Table 4). Circulating miRNA 150 also play a role in clinical prognoses and could be used to molecularly monitor disease evolution as a new prognostic factor in CLL [154].

Acute myeloid leukemia is the most common acute leukemia in adults. Levels of C-miRNAs (miR-10a-5p, 93-5p, 129-5p, 155-5p, 181b-5p, 320d and miR-335) have been shown to be up-regulated in the plasma of AML patients (Table 4).

Several studies were carried out in diffuse large B cell lymphoma that constitutes a part of adult non-Hodgkin lymphoma. This kind of lymphoma is very heterogeneous because it develops in different sites of the human body [155]. The concentration of miRNAs has been found to be deregulated, with high levels of miR-15a, 16, 21, 155 and miR-210 (Table 4) and levels of C-miR-21, 29, 34a, 155 and 210 were found to be up-regulated (Table 4).

Gastrointestinal oncology

Gastrointestinal oncology includes different types of cancer such as colorectal cancer, gastric cancer, hepatocellular carcinoma and pancreatic cancer.

These kinds of cancer have multiple etiologies resulting from genomic modifications, that can be largely worsened from inappropriate lifestyles such as incorrect diet (low fiber foods, red meat, greasy foods) as well as smoking and alcohol abuse. It is furthermore appropriate to include also infectious diseases (hepatitis).

Colorectal cancer is one of the most frequent tumors worldwide, especially in industrialized countries. Actually, there are different ways to investigate the presence of this cancer such as the fecal occult blood test and endoscopic biopsy, the latter, a very invasive procedure is reserved for high-risk individuals. From studies in which miRNAs' involvement has been identified in the generation and progression of this type of tumor, several of them have also been identified in serum and plasma. Among these it can be distinguished that the levels of 37 C-miRNAs have been found to be up-regulated (Table 4) while the levels of nine miRNAs were found to be down-regulated (Table 4). Another group of two C-miRNAs were found to be disregulated (Table 4), and can be used in the future to define a signature of tumor presence and stage [156].

Gastric cancer represents another leading cause of cancer-related deaths worldwide. From the histological viewpoint, two principal types of gastric cancer can be distinguished: intestinal adenocarcinoma and diffuse adenocarcinoma. The etiology of gastric cancer is again associated with genetic and other factors, including diet. It is also demonstrated that infection with *Helicobacter pylori* is crucial for the development of the disease. In the serum and plasma of gastric cancer patients, miRNAs represent an alternative marker for diagnosis. It has been demonstrated that levels of 23 C-miRNAs were found to be up-regulated in patients with gastric cancer (Table 4) and three miRNAs were found to be down-regulated (Table 4). Finally, circulating miRNAs could be used as a follow-up in patients with gastric cancer: serum miR-203 and 18-a have the potential to serve as non-invasive biomarkers for prognosis and to predict metastasis in patients with this type of cancer [157, 158].

Hepatocellular carcinoma is the most prevalent primary liver cancer, with a wide geographic distribution. The most common risk factors associated with this type of carcinoma are the exposure to hepatitis viruses (HBV and HCV), cirrhosis, liver diseases like hemochromatosis and Wilson's disease, alcohol abuse, smoking and aflatoxine exposure. This type of carcinoma shows a deregulation of miRNAs that can be detected in plasma, serum and urine: it has been noticed that levels of 16 C-miRNAs were found to be higher than the healthy controls (one of which is present in urine) (Table 4) while three miRNAs were found to be lower (one of which is present in urine) (Table 4). A third group represented by two miRNAs were found to be deregulated (Table 4), furthermore, hepatocellular carcinoma patients treated with trans-arterial chemoembolization (TACE) could be monitored during follow-up by measuring levels of plasma miRNA 122, as high expression levels of this circulating miRNA seem to be associated with early TACE refractoriness [159].

Pancreatic cancer is one of the deadliest cancer types, accounting for approximately 46,000 new diagnoses and 40,000 deaths annually. Despite the advancement in medical and surgical managements, pancreatic cancer exhibits the poorest prognosis of all solid tumors, with a median survival of 6 months and an overall 5-year survival rate of 5% [160]. To date, by the time a definitive diagnosis is reached, most pancreatic cancer patients (80%) have locally advanced or metastasized disease, displaying a mean survival of less than 1 year [161].

Actually, there are no screening recommendations for pancreatic cancer and it is clear that primary prevention is of the utmost importance in diagnosis. The blood

based-biomarkers carbohydrate antigen 19-9 (CA19-9), carcino-embryonic antigen [162] and/or genetic markers such as *KRAS* and *p53* [163] are not recommended for screening and diagnosis, because of their poor sensitivity and specificity, while they are only used in patient follow-up. Because it is known that pancreatic cancer is characterized by common and frequent genetic mutations, such as *KRAS* (>90%), *TP53* (>50%), *SMAD4* (>60%) and *CDKN2A* (>80%) genes, as crucial regulators of gene expression, miRNAs represent a new promising class of eligible non-invasive biomarkers [164, 165]. For the first time, 25 miRNAs that allowed to correctly discriminate 90% of pancreatic cancer from normal pancreas has been identified [166]. Since then several studies have been conducted to identify diagnostic classifier miRNAs in pancreatic cancer. Ren and Yu, this year summarized in a review paper, the main miRNAs isolated from the serum or plasma and pancreatic juices with a potential role not only in the diagnosis of pancreatic cancer, but also in the prognosis and chemoresistance [167]. In particular, miR-21, 155, 196 and miR-210 are demonstrated to be consistently deregulated in pancreatic patients [168]. miR-192, also showed higher expression levels in blood samples from pancreatic cancer patients, exhibiting high sensitivity (76%) and specificity (55%) [169]. Furthermore, Li et al. demonstrated that miR-1290 can discriminate between the sera of patients with low stage pancreatic cancer from the sera of controls better than CA 19-9 tumour marker [170]. Debernardi et al. demonstrated for the first time, the utility of miRNA biomarkers for non-invasive and early detection of pancreatic cancer in urine specimens. They proved that the combination of miR-143 with miR-30e accurately discriminates between pancreatic cancer patients (stage I) and healthy controls [171]. Another review paper of interest in this context is that of Sun et al. The authors summarized how miRNAs regulate core signalling cascades genetically altered in 67%–100% of pancreatic cancer patients (e.g. apoptosis; control of G1/S phase transition; KRAS, Hedgehog, transforming growth factor-b and Wnt signalling) that contribute to oncogenesis and progression of pancreatic cancer [172].

Breast cancer

Breast cancer is the most commonly diagnosed cancer in women. The genetic background of breast cancer patients includes *BRCA* gene mutations. *BRCA1* and *BRCA2* belong to a class of genes known as tumor suppressors. In normal cells *BRCA1* and *BRCA2* prevent uncontrolled cell growth and contribute to ensure the stability of the cell's genetic. It is clear that mutations in these genes increase the risk of breast cancer. The diagnosis, actually, is based on ultrasound and mammography (this one exposes the patient to ionizing radiation) as well as breast self-examination and serological markers such as CA15.3 and CEA, especially employed in following-up breast cancer patients. It transpires that available biomarkers are only applicable to advanced breast cancer and biomarkers for early diagnosis are lacking. Therefore C-miRNAs could represent a novel class of early biological markers. In fact, miRNAs have been identified in the serum, plasma and milk of breast cancer patients and different studies have shown that C-miRNA levels are altered in breast cancer, precisely 38 C-miRNAs are up-regulated (Table 4), nine C-miRNAs are down-regulated (Table 4) and seven are deregulated (Table 4). It is clear that C-miRNAs could serve as promising markers for breast cancer diagnosis and prognosis, for example, miR-182 and miR-375 could be potential noninvasive markers used for the follow-up of these patients [173].

Ovary

Epithelial ovarian cancer is another common cancer in women and the most common cause of cancer death from gynecologic tumors worldwide. Epidemiological studies identified multiple exogenous and endogenous risk factors for ovarian cancer development such as the inflammatory stromal microenvironment. The interaction between the microenvironment, genetic polymorphisms and different epithelial components such as endosalpingiosis, endometriosis and ovarian inclusion cyst in the ovarian cortex may induce different genetic changes identified in the epithelial component of different histological types of ovarian tumors [174]. Evidence suggests that ovarian cancers can progress by two steps: both through a stepwise mutation process (low-grade pathway) and through greater genetic instability that leads to rapid metastasis without an identifiable precursor lesion (high-grade pathway) [175]. From the viewpoint of diagnosis, actually, there is no reliable method to screen for ovarian cancer and the majority of cases are diagnosed with advanced disease. For this reason, it is important to identify new biomarkers that could help in early diagnosis and prognosis. In fact, it has been noticed that altered levels of C-miRNAs can be detected in the serum of epithelial ovarian cancer patients, and especially the levels of six C-miRNAs were found to be

up-regulated while two miRNAs were found to be down-regulated (Table 4).

Prostate

Prostate adenocarcinoma is the second most diagnosed malignancy in men worldwide and one of the most common causes of cancer death worldwide. The risk factors include age, ethnicity, geographic origin, family history of prostate cancer and genetic factors. Early diagnosis is based on the measurement of prostate-specific antigen (PSA) in combination with clinical examination. It is clear that early diagnosis is a priority in the treatment of this pathology. Thus C-miRNAs can represent a viable alternative [176], they have been detected in different fluids such as serum, plasma, urine and ejaculate and also viral miRNAs have been analyzed in prostate cancer because papilloma virus and herpes simplex virus 2 have been suggested to play roles in prostate cancer development.

For this reason, in prostate cancer patients a number of miRNAs have been found to be deregulated with respect to healthy patients, even by epigenetic modifications [177]. Among the identified miRNAs, different groups can be distinguished depending on their deregulation or the containing matrix. In particular, 41 C-miRNAs are up-regulated: two in urine, one in urine supernatant, one in urine sediment and two in ejaculate (Table 4); five C-miRNAs were down-regulated, thereof two in urine and one in urine sediment (Table 4). Finally, two C-miRNAs were found to be disregulated (Table 4).

Lung

Lung cancer is the most common cause of cancer death worldwide. The classification includes two different types of lung cancer: “small cell lung cancer” that is less common and “non-small cell lung cancer” (NSCLC), the most common epithelial cancer.

NSCLC is a tumor with a very poor prognosis and the most prevalent subtype is represented by adenocarcinoma. The etiology of this tumor derives principally from tobacco smoking but also other risk factors may contribute to the development of this type of cancer (air pollution, exposure to chemicals, etc.). The diagnosis is based on biopsy and radiography, but they cannot ensure an early diagnosis; in fact diagnosis is often late and patients are generally treated with surgical resection.

Currently there is no validated screening method for lung cancer and, for this reason, it is important to identify an ideal class of biomarkers for lung cancer early diagnosis. Different studies have shown that C-miRNA levels change depending on the stage of NSCLC and they are found in different types of fluids such as serum and plasma but also in pleural effusion, sputum and bronchoalveolar lavage. In particular it has been demonstrated that 28 C-miRNAs are up-regulated (Table 4) and 36 are down-regulated, of which four are found in pleural effusion (Table 4). Another group of five C-miRNAs were found to be deregulated (Table 4) and eight are present in sputum and bronchoalveolar lavage (Table 4). In view of the mentioned, they could be a novel class of biomarkers in NSCLC diagnosis and prognosis. It would also be advisable to implement studies in order to use circulating miRNAs in following-up patients with NSCLC.

Conclusions

In conclusion, C-miRNAs represent promising biomarkers for the screening of disease using body fluids instead of standard biopsies. Indeed, the production of these molecules generally can be revealed in the early stages of physiological processes of clinical interest, as their transcription is driven by factors directly involved in initial processes of cell modification, inflammation or cell transformation. However, the presence of several variables, such as the detection in plasma or serum, centrifugation parameters, anticoagulants used for blood collection, methods for RNA extraction and amplification, as well as different methods of patients’stratification, determine a variability in the detection of miR expression levels. Indeed, a robust validation of methodology to detect them needs to be standardized before introducing it as a routine laboratory test [73] to guide clinical decision making., Moreover, standardized systems of big data analysis and management should be set up for data interpretation [178], the development of neural network based software could help the interpretation of the miRNA signature of specific patients [179].

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