Published in final edited form as:
Clin Chem. 2014 September ; 60(9): 1158–1173. doi:10.1373/clinchem.2013.216044.

Noninvasive Micromarkers

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Abstract

BACKGROUND—The recent revolutionary advances made in genome-wide sequencing technology have transformed biology and molecular diagnostics, allowing new sRNA (small RNA) classes to be discovered as potential disease-specific biological indicators. Cell-free microRNAs (miRNAs) have been shown to exist stably in a wide spectrum of body fluids and their expression profiles have been shown to reflect an assortment of physiological conditions, underscoring the utility of this new class of molecules to function as noninvasive biomarkers of disease.

CONTENT—We summarize information on the known mechanisms of miRNA protection and release into extracellular space and compile the current literature on extracellular miRNAs that have been investigated as biomarkers of 20 different cancers, 11 organ damage conditions and 10 diverse disease states. We also discuss the various strategies involved in the miRNA biomarker discovery workflow and provide a critical opinion on the impediments faced by this advancing field that need to be overcome in the laboratory.

SUMMARY—The field of miRNA-centered diagnostics is still in its infancy, and basic questions with regard to the exact role of miRNAs in the pathophysiology of diseases, and the mechanisms of their release from affected cells into biological fluids are yet to be completely understood. Nevertheless, these noninvasive micromarkers have immense potential in translational medicine

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Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

Authors' Disclosures or Potential Conflicts of Interest: Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:
Employment or Leadership: V.S. Vaidya, Harvard Program in Therapeutic Sciences.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: None declared.
Research Funding: V.S. Vaidya, National Institutes of Health/National Institutes of Environmental Health Sciences–Outstanding New Environmental Scientist Award (ES017543) and an Innovation in Regulatory Science Award from the Burroughs Wellcome Fund.
Expert Testimony: None declared.
Patents: None declared.

not only for use in monitoring the efficacy and safety of therapeutic regimens but also to guide the diagnosis of diseases, to determine the risk of developing diseases or conditions, and more importantly, to inform treatment options.

It has been only 2 decades since the discovery of the first microRNA (miRNA),[3] *lin-4*, as a heterochronic gene in the *Caenorhabditis elegans* developmental pathway (1). The sequence of *let-7*, which was discovered soon after (2), was found to be conserved across a wide range of phyla, being expressed at the later stages of development in all species, indicating temporal conservation as well (3). Since then, the research in this field has burgeoned, with over 21 000 sequences currently discovered in a total of 193 species. Today, we know miRNAs to be a family of small noncoding RNA molecules of approximately 22 nucleotides in length that regulate gene expression. In the human genome, 52% of miRNAs are located intergenically, and 40% and 8% are located within introns and exons, respectively (4).

miRNAs are transcribed in the nucleus by RNA polymerase II as primary transcripts called pri-miRNAs, which are further processed by the complex formed by RNase III endonuclease Drosha with DGCR8 protein (Pasha) into 70-nucleotide–long stem-looped precursor miRNAs (Fig. 1A) (5). The precursor miRNA (pre-miRNA) is exported to the cytoplasm via Exportin 5 in a Ran GTP– dependent manner, where it is cleaved by Dicer, another RNase III endonuclease which associates with *trans*-activation response (TAR) RNA-binding protein (TRBP) to produce an miRNA–miRNA* duplex comprising the functional fragment (miRNA) and a low-abundance fragment (miRNA*). Normally the functional miRNA that enters the RNA-induced silencing complex (RISC) originates from the strand with the least stable 5′ end, which lends asymmetry to the complex. The typical recovery ratio for miRNA and miRNA* from the miRNA–miRNA* duplex is about 100:1, except in the rare cases in which both arms of the precursor are equally stable and have similar recoveries (6).

The functional miRNA is incorporated into the RISC assembled by Dicer/TRBP and an Argonaute protein (AGO-2), which functions as the catalytic endonuclease (5). Near perfect complementarity in the 3′ untranslated region of the mRNA to the seed sequence (2– 8 nucleotides) in the 5′ end of the miRNA leads to cleavage of the target mRNA. On the other hand, an imprecise complementarity assures translational repression of some form, which leaves the mRNA target intact (Fig. 1A). miRNAs are predicted to form pairing with nearly 60% of all protein-coding genes (7) and are known to impact diverse cellular processes, such as proliferation, differentiation, and cell death (5).

Mechanisms of miRNA Protection in Circulation

Recently, cell-free miRNAs have been discovered to exist in circulatory, secreted, and excreted body fluids. These extracellular miRNAs are physiologically functional and have been shown to exert gene silencing in downstream target cells (8). The surprising resistance of miRNAs to RNA-degrading enzymes in circulation is a result of several as yet unearthed

[3]Nonstandard abbreviations: miRNA, microRNA; pri-miRNA, primary miRNA transcripts; pre-miRNA, precursor miRNA; TAR, *trans*-activation response; TRBP, TAR binding protein; RISC, RNA- induced silencing complex; AGO-2, Argonaute protein; nSMase2, neutral sphingomyelinase 2; FFPE, formalin-fixed paraffin-embedded; CSF, cerebrospinal fluid; qPCR, quantitative PCR; AMI. acute myocardial infarction; ESRD, end-stage renal disease.

mechanisms of protection (Fig. 1B). miRNAs in their naked form are not innately resistant to RNase activity owing to their size or structure, because artificially introduced synthetic *Caenorhabditis elegans* miRNAs are rapidly degraded in plasma, whereas endogenous miRNAs remain unaltered (8, 9). The mechanism that has been most investigated so far is the encapsulation of miRNAs into vesicular bodies such as exosomes (10–100 nm) and microvesicles (0.1–1 μm). The first report was in 2007 by Valadi et al., who showed that exosomes shed by human and mouse mast cells contain miRNAs that are deliverable in a functional form to recipient cells (10). Kosaka et al. showed in HEK293 cells that the packaging of miRNAs into exosomes is a ceramide-dependent mechanism under the regulation of neutral sphingomyelinase 2 (nSMase2) (8) and the process has been shown to be ATP dependent in a separate study (11). Exosomal miRNAs have been discovered in human saliva, breast milk, and urine apart from plasma (12–14). Another class of vesicles, apoptotic bodies (0.5–2 μm), have been demonstrated to carry miRNAs from apoptotic endothelial cells during atherosclerosis to neighboring cells to induce survival and growth signals (15). Experiments by Wang et al. on HepG2 and A549 cell lines indicate that following serum deprivation there is a sharp increase in the amount of extracellular miRNAs with a concomitant drop in intracellular concentration, which recovers after a few hours (11). This leads to the assumption that miRNAs to be exported are derived from a presynthesized pool. Apart from the vesicular fraction, miRNAs have also been identified in the supernatant after ultracentrifugation processes to isolate the vesicles (11, 16). This suggests that there are other forms of protection, and they have been shown to include conjugation with a variety of proteins, namely, nucleophosmin 1 (11), HDL (17), and AGO-2 (16, 18). Delivery of miRNAs conjugated with HDL to recipient cells is under the regulation of scavenger receptor class B type 1 (17). Interestingly, loading of HDL with miRNAs appears to be repressed by nSMase2, which indicates that exosomal secretion and HDL incorporation of miRNA may be opposing cellular mechanisms (17), although this has never been proven in the same cell line.

The clearance of miRNAs from circulation is another important mechanistic aspect that remains incompletely explored. Although the half-life of miRNAs has not been measured in extracellular fluids, it is likely that they persist for a long time because of the stable miRNA–protein/lipid complexes. Possible routes of clearance from circulation include miRNA-specific degradation pathways, filtration by the kidneys, or removal by the hepatic system. However, Weber et al. report that there is little correlation between the plasma and urinary profiles of miRNAs, indicating either that the kidneys are not involved in physiological clearance or that miRNAs filtered into the urine are degraded rapidly (19). Further evidence for the former possibility comes from a study which showed that decreased circulating concentrations of miRNAs in patients with chronic kidney disease was not associated with an increase in the urinary concentration (20).

Circulating miRNAs as Biomarkers

miRNAs have not only been shown to play critical regulatory roles in health and disease, but have also been investigated as biomarkers for detecting and predicting disease progression, as well as for therapeutic intervention. Abundant expression, lower complexity, stability in various detection matrices, and amplifiable signals are some qualities that make extracellular

miRNAs attractive candidates as biomarkers reflecting a variety of pathophysiological conditions (21–24) (Table 1). Although proteins have significantly advanced our understanding of biomarker science, there are several drawbacks concerning methods of discovery and detection, availability and stability. Classical immunoaffinity-based methods for measurement of protein biomarkers detect antigen–antibody complex formation in various platforms. These techniques, which include ELISA, rely heavily on the specificity and concentration of the antibody used (25). Platforms that provide multiplexing to measure several antigens in one assay run into problems of diverse range of abundance resulting in the inability for uniform amplification. Posttranslational modifications like glycosylation, acetylation, and lipidation occur frequently and further increase the complexity of the proteome (26). The presence of proteolytic enzymes in nearly all biological samples used for biomarker analysis renders the need for careful collection and preservation of samples. Detection of proteins from preserved specimens such as formalin-fixed paraffin-embedded (FFPE) sections requires extensive antigen retrieval and extraction treatments (27).

Since the discovery of stably expressed miRNAs in serum in 2008 (28), these small molecules have been discovered in a plethora of other biological fluids such as urine, saliva, breast milk, amniotic fluid, tears, feces, seminal and vaginal fluid, sputum, and cerebrospinal fluid (CSF) (Fig. 1C). The most striking advantage of miRNAs over all other molecules that are in use as biomarkers is their stability across a wide range of physiological and storage conditions. miRNAs in serum have been shown to resist degradation due to extreme temperature and pH fluctuations, freeze–thaw cycles, extended storage of samples, and RNase A digestion (9, 24, 29). Several groups have demonstrated successful isolation of miRNAs from a wide range of sample types including FFPE sections (30), bone marrow aspirate slides (31), and bone marrow core biopsies (32). miRNAs are ubiquitously present and are conserved in mature sequence across several organisms (33), and yet within a species, their expression levels are reproducibly consistent among individuals. The absence of post-transcriptional modification of miRNAs is another attractive feature that confers uniformity and lowers the complexity of the system. Several miRNAs have unique cellular or tissue localization or disease-specific expression that lends specificity to their roles as biomarkers.

Pioneering studies characterizing miRNAs as fluid-based biomarkers have typically used an individual miRNA or a panel of miRNAs to distinguish between cohorts, and statistical measures such as the areas under ROC curves indicate that they have diagnostic accuracies ranging from 70% to 95% (34, 35). These proof-of-concept experiments have laid the foundation on which future miRNA research has developed, taking forward the theme of characterizing entire miRNA profiles to specific diseases (36). An miRNA-based marker will ensure specificity because by virtue of sheer numbers, unique expression signatures can be identified for disease initiation, progression, prognosis, and classification. The wide batteries of tests now being conducted for the same purpose involve measurement of antigens, proteins, lipids, and enzymes and are hindered by shortcomings such as high detection thresholds, low throughput, delayed expression, and variable sensitivities. Although a universally standardized procedure of RNA isolation and miRNA detection is awaited, amplification-based techniques such as quantitative PCR (qPCR) have been shown to be simple, fast, reproducible, and cost-effective.

Some studies have examined the variation of miRNAs in the healthy population, because this is important in interpreting the results from different disease states and biological fluids. Weber et al. measured the miRNA composition of 12 different body fluids in 5 healthy samples and determined that saliva, breast milk, and seminal fluid have larger numbers of detectable miRNA species in comparison with urine or CSF (19). On the basis of commonly expressed miRNAs, these investigators were able to cluster the fluids into 2 major groups, with plasma having a profile distinct from most other fluid types. We analyzed the temporal variation in a select number of candidate miRNAs in the urine samples of 29 healthy individuals (36) and observed that although there was considerable background variation in the healthy samples, the miRNA concentrations were significantly increased in disease conditions. This ubiquitous and variant dispersion of the miRNA population suggests their importance in maintaining normal physiological functions in a variety of organ systems.

miRNAS AS BIOMARKERS OF CANCER

The knowledge that miRNAs are released outside the cell and exist stably in various body fluids has received considerable attention from the field of cancer biology, where the gold standards of detection are usually invasive, and the necessity for extracellular and noninvasive sources of biomarkers is growing urgent (Table 2). Most of the studies have been performed on serum/ plasma, but some groups have examined other fluids relevant to diseases, such as saliva for oral carcinoma (37), urine for renal (38) and bladder cancer (39), sputum for lung cancer (40), and CSF for lymphoma of the central nervous system (41) and glioblastoma (42). One of the first attempts to identify miRNA biomarkers in a noninvasive body fluid was made in 2008, by comparing the concentrations of 3 miRNAs known to be associated with tumors in the serum of patients with diffuse large B-cell lymphoma with concentrations in healthy controls (28). It was found that miRNA concentrations are higher in the patient sera and specifically that high miR-21 concentrations indicated relapse-free survival. Around the same time, another group showed that miRNAs are present in a very stable and detectable form in human plasma and can distinguish prostate cancer xenograft mice from controls (9).

One of the most common study designs that many groups have followed is to screen for differentially regulated miRNAs in paired cancerous and noncancerous tissues. Thereby, individual miRNAs or a panel is selected and further examined in the chosen fluid matrix (43, 44). Another widely used approach is to perform the initial screen in pooled samples and then confirm the results in individual samples (45). A few groups have studied the dysregulation of miRNAs in mouse models and translated the results to humans (46) or have performed in vitro experiments in cancerous cell lines before expanding the study to patient samples (47). An appropriate inclusion in the study design is to evaluate the miRNA expression before and after tumor removal as a prognostic indicator of disease regression (48). Friedman et al. used serum miRNAs to predict the recurrence of melanoma after surgical resection of the tumor (49).

Several studies highlight the specificity of candidate miRNAs as biomarkers of cancer by comparing their concentrations in closely related cancer types or disease states. For example, while identifying potential serum miRNA biomarkers for pancreatic ductal

adenocarcinoma, Kong et al. used chronic pancreatic cancer samples to validate the specificity of miR-196a (50). Hanke's group compared urine samples from patients with low- and high-grade bladder cancer to noncancerous urinary tract infection in their study (51). Some studies highlight the prognostic ability of miRNAs to classify different grades or stages of cancer. Roth et al. compared the concentrations of serum miRNAs in primary vs metastatic breast cancer patients (47), while a study by Asaga et al. demonstrated that patients with advanced stages of breast cancer have significantly higher circulating miR-21 than those in earlier stages (43).

miRNAS AS BIOMARKERS OF ORGAN DAMAGE CONDITIONS

miRNA-based diagnostics have also been investigated in various organ damage conditions, some of which are summarized in Table 3. The spectrum of cardiovascular diseases has been a prime focus of miRNA bio-marker research. miR-1 and -499 have been proposed and verified as biomarkers of acute myocardial infarction (AMI) that are comparable to conventional standards (52). Cheng's group employed a rat model of AMI and found serum miR-1 to increase early, peaking at 6 h, and to be well correlated with the size of the infarct (52). On translating their findings to humans, they observed serum miR-1 patterns to correlate with the conventional serum creatine kinase-MB. In both rats and humans, urinary miR-1 was found to increase significantly at early time-points after AMI as well, peaking at 24 h (53). Heart failure, coronary artery disease, and hypertension are other heart diseases that have been extensively worked on (52).

Urine has been one of the most widely used matrices for biomarker discovery in the context of kidney diseases. miR-210 has been proposed as a plasma and urinary biomarker for acute and chronic forms of injury and end-stage renal disease (ESRD) (20, 34). The urinary form could predict acute allograft rejection and has the potential to be a noninvasive monitor of graft function and susceptibility to medication (54). miR-155 deregulation in urine and plasma has also been studied in acute and chronic injury and ESRD (20, 55).

One of the most common forms of liver damage, acetaminophen toxicity, was found to upregulate miR-122 in the plasma in several rodent and human studies (21, 56). miR-122 and miR-192, both liver-enriched species, were shown to have an earlier and dose-dependent response to drug administration and are translatable, making them very suitable candidates for toxicity monitoring of large-scale drug and compound screening (21). miR-122 has also been shown to be immensely deregulated in nonalcoholic fatty liver disease and chronic hepatitis C (57).

miRNAS AS BIOMARKERS OF DISEASE CONDITIONS

Apart from cancer and organ damage conditions, there are many other disease states in which miRNAs have been investigated as biomarkers, and a few such studies are listed in Table 4. Zampetaki et al. discovered that the concentrations of 5 miRNAs in the plasma of patients with diabetes differed from those in plasma from healthy individuals. This dysregulation was then confirmed in about 200 samples from individuals with or without diabetes, and miR-126 evaluation was further extended to 822 different samples (58). Another clinically relevant condition that necessitates rapid and early diagnosis and lacks an

ideal biomarker is sepsis. It was shown that plasma miR-150 is significantly lower in sepsis patients and that the concentrations correlate with the severity of the disease (59). Serum miR-146a and -223 concentrations were both found to be lower in sepsis patients than in healthy controls (60).

There have been studies of other disease conditions, including multiple sclerosis (61), HIV encephalitis (62), and biopolar disorder (63) for the use of miRNAs in various body fluids as novel and noninvasive biomarkers.

Tools and Techniques Used in miRNA Biomarker Research

ISOLATION

The conventional methods of RNA isolation include phenol– chloroform extraction with commercial reagents or glass–fiber column– based extraction from various manufacturers (Fig. 2). Techniques for exosome isolation and preconcentration include commercial precipitation reagents, ultracentrifugation, chromatography, and Dynabeads (13, 64). Yoo et al. have described an innovative method to isolate miRNAs directly from exosomes using immunoaffinity magnetic beads (65). Quantification of the extracted RNA is usually performed by spectophotometric analysis on the Nanodrop. Agilent's microfluidics platform can be used to assess the quantity and quality of total RNA as well as low molecular weight species separately. Studies that specifically isolate exosomes use transmission electron microscopy (TEM) to study the vesicle structure and integrity or perform immunoblot analysis for exosome-specific proteins such as CD63 and TSG101 (14).

DISCOVERY

Profiling—Numerous companies offer miRNA profiling using amplification-based as well as hybridization-based platforms (Fig. 2). Although hybridization systems work well for profiling in tissues and avoid the amplification bias, the low RNA yield from extra-cellular fluids poses a problem in this context. Thus, amplification-based profiling techniques that provide an additional option of preamplification may be the best choice for systems such as urine, which have a very low RNA abundance to begin with. In addition to commercially available platforms, several groups have developed other techniques for expression profiling (66).

Sequencing—Next generation sequencing of nucleic acids is increasingly used to identify new species-specific and tissue-specific miRNAs. The technologies that are most commonly used are 454 pyrosequencing, Solexa sequencing, and ABI solid sequencing. HeliScope single-molecule sequencing has also been demonstrated for the sequencing of small RNAs (Fig. 2). There are comprehensive reviews detailing the complete workflow of deep sequencing and the advantages and drawbacks of each method (67).

DETECTION

The detection of single or multiple miRNAs obtained after profiling/sequencing is usually performed by qPCR, Northern blotting, or in situ hybridizations. Traditional qPCR is performed by using stem-looped RT primers (like Taqman), an miRNA-specific forward

primer, and a universal reverse primer for PCR. Another strategy is to add a polyA tag to the RNA and use an oligo-dT primer for RT and specific forward and universal reverse primers for PCR (like Qiagen). Improvisations include polyuridylating and using a polyA stem-loop primer during RT for better specificity and convenience (68). In situ hybridization is another commonly used method of detection, which is improved in selectivity by using locked nucleic acid probes (69). De Planell-Saguer et al. have developed a unique technique to simultaneously detect miRNAs and proteins from FFPE sections and cell cultures (70). This could be especially useful in miRNA–target interaction studies.

Several novel and original methods of detection have been developed, many of them having a fluorescence-, electrochemical-, or optical-based detection step. These protocols aim at expanding the utility of miRNAs into diagnostic tools that can be efficiently incorporated into a clinical setting. Many of these assays allow for rapid quantification, have improved sensitivity and specificity of detection, and reduce the number of amplification steps required before detection. For instance, a scheme put forth by Schoch et al. isolates, concentrates, and quantifies small RNAs from cell lysates using on-chip isotachophoresis (71). Strategies of detection vary from using simple molecular beacons (72), graphene-oxide– based fluorescence quenching (73), and enzymatic luminescence (74) to different nanoparticle-based probes like silver (75), gold (76), and carbon (77). More complex methods involve different forms of electrophoresis, like isotachophoresis (78) and capillary electrophoresis (79), circular exponential amplification (80), or isothermal amplification (81) and various forms of hybridization (82).

Challenges

Although the use of miRNAs as biomarkers has been proposed and verified in various disease models, there are several impediments that need to be overcome before they can be scaled up into clinical translational markers.

ORIGINS

The origins of extracellular miRNAs remain obscure and could potentially affect the significance of the results. For example, it has been shown that tumors specifically secrete exosomes that carry miRNAs into the circulation (83), which indicates that extracellular miRNAs have an associative role in the initiation, progression, or metastasis of the tumor, making them relevant choices for biomarkers. A recent study indicated that miRNAs present in serum and saliva are for the most part concentrated in exosomes (14). However, studies by Arroyo et al. and Turchinovich et al. assert that miRNAs in circulation are principally found in association with protein complexes and not vesicles (16, 18), raising the possibility that the detected miRNAs are remnants of dead cells protected by stable protein complexes (16).

BIOMATERIAL SELECTION

It is not clear whether choosing serum or plasma contributes any difference to the results. Although Mitchell’s group reported that there is no difference in abundance between serum and plasma for selected miRNAs (9), a recent study showed that the total concentration of

RNAs as well as detectable miRNA abundance is higher in serum than in plasma (84). The group suggests that coagulation may trigger the release or secretion of miRNAs, or RNA extrusion due to cell lysis may contribute to the effect. Another important factor to take into consideration while dealing with clinical plasma/serum samples is the extent of hemolysis. Kirschner et al. demonstrate that miR-16 and -451, commonly used as normalizing factors, are present in substantial amounts in red blood cells and the extent of hemolysis causes considerable variation in their concentrations between samples (85).

ISOLATION TECHNIQUES

Differences in RNA stabilization systems for blood may cause differences in yields and purity of the RNA, although they do not seem to affect downstream qPCR applications (86). Isolation of RNA is a crucial step, and yield differs with technique, introducing considerable variability in qPCR and microarray-based results (87). Several studies have been performed that compare TRIzol extraction to column-based isolation and the use of exosome precipitation protocols to ultracentrifugation in a wide variety of samples (29, 87). The discrepancies in the conclusions indicate that sample type, protocol, reagent variation, and quantification methods influence the quality and quantity of RNA isolated. As with mRNA analysis, different microarray platforms used for quantification of miRNAs have inconsistencies in their results, and there is variation between microarray and qPCR/Northern blot validation data (88). Even with deep sequencing strategies, the protocols for library preparation and selection of the sequencing platform were found to influence the findings (89).

NORMALIZATION

The lack of a standardized housekeeping miRNA that is consistent between different tissues and extracellular fluids and between disease states is a crippling factor in their putative use as biomarkers (90). Alternative means of normalizing include synthetic miRNA spiked into the sample, which serves as a good technical control, or the use of equal amounts of starting total RNA as quantified on a spectrophotometer. However, a spiked miRNA will not account for endogenous differences in miRNA concentrations due to disease conditions, and there are studies that indicate that the correlation between total RNA amounts and miRNA concentrations is low (84). Meyer et al. provide an analysis of the utility of 7 different methods of normalization for microarray data (91). The most commonly used mode of normalization for microarray data is to select a panel of endogenous invariant miRNAs across the cases and controls for a particular disease condition and profiling platform (92).

Conclusions

The field of miRNA-centered diagnostics is still in its infancy and these micromolecules have immense potential not only to be developed into sensitive, specific, and robust markers of organ damage and disease conditions but also to monitor disease progression and efficacy of treatment. The National Center for Advancing Translational Sciences, which is a part of NIH, has recently funded a program on extracellular RNA communication that includes miRNAs. Their initiatives are to assess the clinical utility of these molecules as both biomarkers and therapeutic agents and to create healthy reference profiles of extracellular

RNAs present in various body fluids. An industry consortium comprising Pfizer and Eli Lilly and overseen by the Predictive Safety Testing Consortium (PSTC) at the Critical Path Institute is examining circulating miRNAs as markers of testicular damage in response to toxicity (93). However, basic questions with regard to their exact roles in the pathophysiology of disease and the mechanisms of their release from affected cells into biological fluids are yet to be answered. miRNAs have been launched as a new generation of biomarkers, and once the various technical and biological hurdles can be overcome, they hold the promise of traversing the biomarker pipeline all the way from preclinical and clinical studies to emerge into the market for consumer use.

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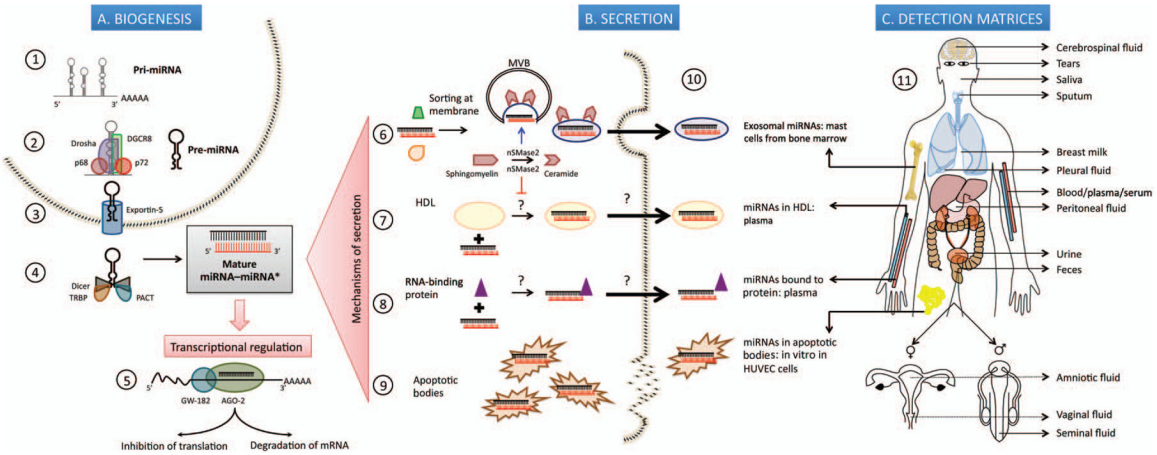

Fig. 1. MicroRNA (miRNA) biogenesis and secretion into extracellular space

(A), Biogenesis. 1. Transcription of pri-miRNAs from genes in the nucleus by RNA polymerase II. 2. Processing of primary transcript into 70-nucleotide (nt)–long pre-miRNA by the Drosha-DGCR8 Microprocessor complex assisted by p68 (DDX5) and p72 (DDX17) DEAD-box RNA helicases that possibly act as scaffolding proteins to recruit cofactors. 3. Export into cytoplasm in a Ran-GTP– dependent manner through Exportin 5. 4. Cleavage of pre-miRNA by RNase III enzyme Dicer, into a 22-nt double-stranded RNA composed of the mature miRNA “guide” strand and the low-abundance miRNA* “passenger” strand; TRBP and protein activator of interferon-induced protein kinase PKR (PACT) are some of the molecules that regulate this step. 5. Incorporation of mature miRNA into the RISC, whose main components are AGO-2 and GW-182; partial complementarity of the seed region of miRNA to the 3′ untranslated region of target mRNAs causes translational repression while complete complementarity leads to degradation of the transcript. (B), Secretion of miRNAs into extracellular space by: 6. Packaging into multivesicular bodies (MVBs) that fuse with the plasma membrane and release as exosomes in a ceramide-dependent pathway positively regulated by nSMase2. 7. Encapsulation into HDL particles, a process which is repressed by nSMase2. 8. Binding to RNA-binding proteins, namely AGO-2 and nucleophosmin 1 (NPM1). 9. Incorporation into apoptotic bodies. 10. Pioneering studies describing exosomal miRNAs isolated from primary bone marrow derived mast cells; HDL-miRNA in human plasma; protein-bound miRNAs in human plasma (AGO-2) and human cell lines (NPM1 in HepG2 and A549); miR-126 from endothelial cell-derived apoptotic bodies in vitro in human umbilical vein endothelial cells (HUVEC). (C), Detection matrices. 11. Depiction of 13 different matrices where extracellular miRNAs have been discovered.

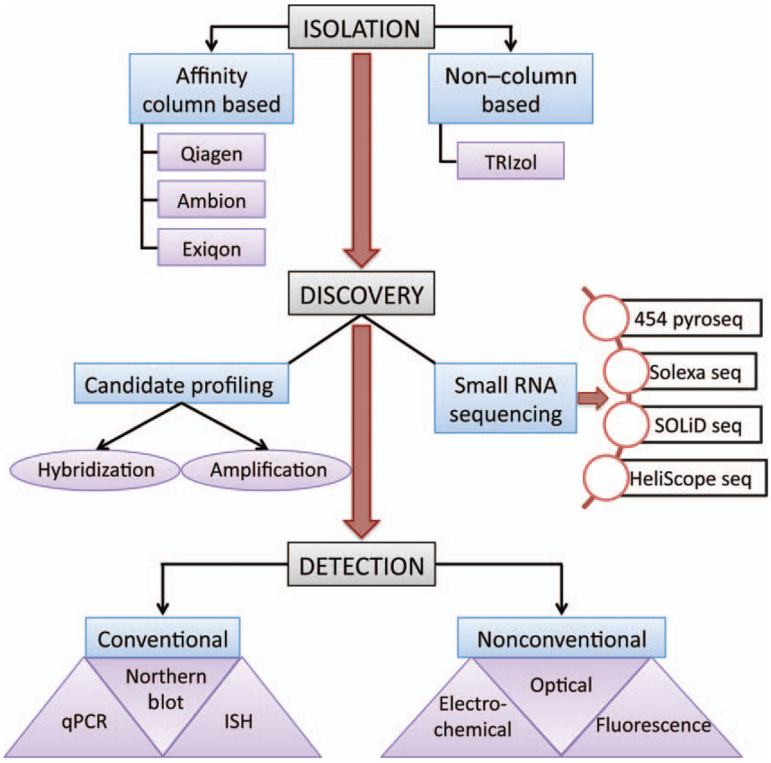

Fig. 2.

Tools and techniques used in miRNA bio-marker discovery in the RNA isolation, candidate discovery, and verification stages. Seq, sequencing.

Table 1

Salient features of miRNAs making them ideal biomarkers.

Sensitive: immediate release before mRNA transcription and protein translation
Specific: tissue- and disease-specific regulation
Dynamic range: approximately 50,000 copies of miRNA/cell
Translational potential: highly conserved with a high degree of inter- and intraspecies homology
Stability: more resistant to degradation than mRNA and proteins and able to be detected in FFPE tissues (although DNA is more stable, it is less tissue specific)
Accessible in a plethora of biological fluids, allowing noninvasive detection
Quantitative by PCR amplification

Table 2

Extracellular microRNAs as biomarkers of cancer.[a]

Disease	Body fluid	Candidate miRNAs	Cohort used			Normalizer	Reference
			Discovery	Confirmation	Evaluation		
Prostate cancer (PC)	Serum	miR-141	1 Control	25 PC, 25 controls	—	*C. elegans synthetic miRNA*	Mitchell et al. (9)
	Serum	Panel of 15 miRNAs	5 PC, 8 controls	—	—	*z*-Score normalization	Sita-Lumsden et al. (94)
	Serum	miR-375, -141	7 Metastatic, 14 localized PC	42 PC, 3 metastatic	71 Primary PC	*C. elegans* synthetic miRNA	Sita-Lumsden et al. (94)
Diffuse large B-cell lymphoma (DLBCL)	Serum	miR-155, -210, -21	60 DLBCL, 43 controls	—	—	miR-16	Lawrie al. (28)
	Serum	miR-15a, -16–1, -29c, -155	20 DLBCL, 20 controls	75 DLBCL, 77 controls	—	*C. elegans* miR-39	Fang et al. (95)
Leukemia	Plasma	miR-92a, -638	2 Acute myeloid leukemia (AML), 7 controls	54 AML, 7 Acute lymphoblastic leukemia, 16 controls	—	Raw data	Tanaka et al. (96)
Pancreatic cancer (PnC)	Plasma	miR-210	11 PnC, 14 controls	11 PnC, 11 controls	—	*C. elegans* miR-54	Ho et al. (97)
	Serum	miR-20a, -21, -24, -25, -99a, -185, -191	Pooled 25 PnC, 25 controls	25 PnC, 25 controls; 95 PnC, 81 controls	82 Chronic pancreatitis (CP); 37 PnC, 32 controls; 55 retrospective	Serum volume	Liu et al. (98)
	Plasma	miR-21, -221, -200b, -200c, -146a, let 7b, let 7d	Pooled 50 PnC, 10 controls	32 PnC, 10 controls	—	miR-16	Ali et al. (99)
Pancreatic ductal adenocarcinoma (PDAC)	Plasma	miR-21, -210, -155, -196a	28 PDAC, 19 controls	—	—	miR-16	Wang et al. (100)
	Serum	miR-196a, -21, -155, -210	35 PDAC, 15 CP, 15 controls	—	—	Raw data	Kong et al. (50)
Breast cancer (BC)	Whole blood	miR-195, let 7a	83 BC, 44 controls	29 BC after surgery	—	miR-16	Heneghan et al. (101)
	Serum	miR-10b, -34a, -155	4 BC cell lines	59 Primary BC, 30 metastatic, 29 controls	—	miR-16	Roth et al. (47)
	Serum	miR-21	14 BC and normal tissue	40 BC (10 each stage), 10 controls	102 BC different stages, 20 controls	miR-16	Asaga et al. (43)
	Plasma	miR-16, -21, -145, -451	5 BC and control plasma,	15 BC before and after surgery, 15 controls	170 BC, 95 different cancers, 100	RNU6B	Ng et al. (102)

Disease	Body fluid	Candidate miRNAs	Cohort used			Normalizer	Reference
			Discovery	Confirmation	Evaluation		
			BC and normal tissue BC and normal tissue		controls; 70 BC, 50 controls		
Ovarian cancer (OvC)	Serum	miR-21, -92, -93, -126, -29a, -155, -127, -99b	9 OvC, 4 controls	19 OvC, 11 controls	—	miR-142–3p	Resnick et al. (103)
	Serum exosome	miR-21, -141, -200a, -200b, -200c, -203, -205, -214	Exosome derived vs tumor derived profile	50 OvC different stages, 10 benign, 10 controls	—	Ambion control miRNA	Taylor and Gercel-Taylor (104)
Gastric cancer (GC)	Plasma	miR-17–5p, -21, -106a, -106b, let-7a	34 GC, 15 controls; 8 GC in plasma and FFPE	10 GC before and after surgery	69 GC different stages	RNU6B	Wang et al. (105)
	Serum	miR-1, -20a, -27a, -34, -423, -5p	Pooled 20 metastatic GC, 20 controls	22 GC, 22 controls	142 GC, 105 controls	Standard curve calibration	Wang et al. (105)
	Plasma	miR-223, -21, -218	10 GC, 10 controls	8 GC plasma and tissue	60 GC, 60 controls	RNU6B	Li et al. (106)
	Serum	miR-221, -376c, -744	Pooled and individual 14 GC, 14 controls	68 GC, 68 controls; 42 dysplasia, 42 controls	58 GC retrospective	ath-miR-159a, profiling; *C. elegans* miR-39, qPCR	Wang et al. (105)
	Serum	miR-378	7 GC, 7 CRC, 10 controls	10 GC, 10 CRC, 10 controls	40 GC, 41 controls; 4 GC tissue	RNU6B	Wang et al. (105)
Colorectal cancer (CRC)	Plasma	miR-92, -17–3p	5 CRC and control plasma; CRC and normal tissue	25 CRC, 20 controls; 10 CRC before and 7 days after surgery	90 CRC, 20 GC, 20 inflammatory bowel diseases, 50 controls	RNU6B	Menendez et al. (107)
	Plasma	miR-29a, -92a	20 CRC, 20 controls	80 CRC, 37 adenomas, 39 controls	20 CRC before and after surgery	miR-16	Menendez et al. (107)
	Stool	miR-92a, -21	88 CRC, 57 polyps, 101 controls	40 CRC and normal tissue	9 and 10 CRC before and after surgery	Standard curve calibration	Wu et al. (108)
	Stool	miR-144*	15 CRC and controls	35 CRC and 40 controls	15 CRC and normal tissue	miR-378	Kalimutho et al. (109)
	Plasma	miR-141	74 CRC different stages, 28 controls	108 CRC, 48 controls	21 Metastatic, 24 localized tumors	*C. elegans* miR-39	Menendez et al. (107)
Non–small cell lung carcinoma (NSCLC)	Serum	miR-21	6 NSCLC and normal tissue	70 NSCLC, 44 controls	—	RNU6B	Liu et al. (44)
Lung adenocarcinoma (LA)	Sputum	miR-21, -486, -375, -200b	20 LA and normal tissue	36 LA, 36 controls	64 LA, 58 controls	RNU6B	Yu et al. (110)

Disease	Body fluid	Candidate miRNAs	Cohort used			Normalizer	Reference
			Discovery	Confirmation	Evaluation		
Squamous lung cell carcinoma (LC)	Sputum	miR-205, -210, -708	15 LC and normal tissue	48 LC, 48 controls	67 LC, 55 controls	RNU6B	Xing et al. (40)
Hepatocellular carcinoma (HCC)	Serum	miR-500	Mouse tissue; 6 liver cancer cell lines	40 HCC and normal tissues	3 HCC before and after surgery	RNU6B, tissue; miR-1, serum	Yamamoto et al. (46)
	Serum	miR-25, -375, let-7f	30 HCC, 30 controls	30 HCC, 30 controls	55 HCC, 50 controls	Serum volume	Li et al. (111)
	Urine	miR-618, -650	Pooled 32 HCC post–hepatitis C virus (HCV), 74 HCV, 12 controls	Individual validation	—	5S rRNA	Abdalla and Haj-Ahmad (112)
	Plasma	miR-21	126 HCC, 30 hepatitis, 50 controls	10 HCC before and after surgery	—	miR-16	Tomimaru et al. (113)
Primary central nervous system lymphoma (PCL)	CSF	miR-21, -19b, -92	23 PCL, 10 other neurological problems	23 PCL, 30 other neurological problems	—	miR-24	Baraniskin et al. (41)
Glioblastoma (GBM)	CSF	miR-10b, -21	19 GBM, 16 breast to brain, 26 breast leptomeningeal metastasis (LM), 28 lung to brain, 4 lung LM, 15 controls	1 GBM, 2 NSCLC temporal study	—	miR-24	Teplyuk et al. (42)
Bladder cancer (BCa)	Urine	miR-143, -222, -452	37 BCa tissue and urine	37 BCa, 37 benign urinary diseases, 20 controls	—	miR-16	Puerta-Gil et al. (39)
	Urine	miR-1224–3p, -135b, -15b	68 BCa, 58 benign urinary diseases	—	—	RNU44, RNU48	Miah et al. (114)
	Urine	miR-126, -152, -182	9 low BCa, 9 high BCa, 9 urinary tract infection (UTI), 9 controls	11 low BCa, 18 high BCa, 7 UTI, 11 controls	—	RNU6B	Hanke et al. (51)
Malignant melanoma (MM)	Serum	miR-221	90 different types of MM, 20 controls	8 MM before, after surgery, at recurrence	—	*C. elegans* miR-54	Kanemaru et al. (115)
	Serum	miR-103, -221, -222, -423, -5p, 199a-5p, -33a, -424	80 MM (discovery) and	10 MM before and after surgery	17 MM before and after recurrence	Raw data	Friedman et al. (49)

Disease	Body fluid	Candidate miRNAs	Cohort used			Normalizer	Reference
			Discovery	Confirmation	Evaluation		
			50 MM (validation) 50 MM (validation)				
Oral squamous cell carcinoma (OSCC)	Plasma	miR-24	43 OSCC and normal tissue	33 OSCC, 10 controls	—	RNU6B, let7a	Lin et al. (116)
	Plasma	miR-31	43 OSCC before and after surgery, 21 controls	9 OSCC - saliva before and after surgery	—	miR-16	Liu et al. (117)
	Saliva	miR-125a, -200a	12 Controls	12 OSCC, 12 controls	38 OSCC, 38 controls	RNU6B	Park et al. (37)
	Saliva	miR-31	45 OSCC, 10 Oral verrucous leukoplakia, 24 controls	22 OSCC before and after surgery	28 OSCC, 17 controls, plasma and saliva	miR-16	Liu et al. (48)
Esophageal squamous cell carcinoma (ESCC)	Serum	miR-10a, -22, -100, -148b, -223, -133a, -127–3p	Pooled 86 nonmetastatic, 55 metastatic ESCC, 40 controls	36 ESCC, 30 controls	113 ESCC, 67 controls	Serum volume	Zhang et al. (45)
	Plasma	miR-21, -375	20 ESCC, 10 controls	8 ESCC before and after surgery	50 ESCC, 20 controls	RNU6B	Komatsu et al. (118)
	Serum	miR-31	45 ESCC and normal tissue; 120 ESCC, 121 controls	64 ESCC before and after surgery	81 ESCC, 120 different tumors, 81 controls	miR-16	Zhang et al. (119)
Renal cell carcinoma (RCC)	Urine	miR-15a	23 RCC and 5 controls tissue	10 RCC before and after surgery	10 RCC, 35 different tumors	5S rRNA	von Brandenstein et al. (38)
	Serum	miR-26a-2*, -191, - 337–3p, -378	25 RCC, 25 controls	117 RCC, 14 benign renal tumors, 123 controls	—	*C. elegans* miR-39	Hauser et al. (120)
	Serum	miR-378, -451	Pooled 15 RCC, 12 controls	90 RCC, 35 controls	—	miR-16	Redova et al. (121)

[a] List of 20 different cancer types in which miRNAs have been evaluated as biomarkers. Each row describes the detection matrix of, study design, and normalization method used. Discovery and confirmation steps were typically performed in a cohort with a smaller sample size to select/verify candidate miRNAs, and evaluation was conducted in an expanded cross-sectional or longitudinal cohort to test the specificity, sensitivity, or early diagnostic capability.

Studies were conducted using human biospecimens unless otherwise stated.

Table 3

Extracellular miRNAs as biomarkers of organ damage.[a]

Disease	Body fluid	Candidate miRNAs	Cohorts used			Normalizer	Reference
			Discovery	Confirmation	Evaluation		
AMI	Plasma	miR-1	93 AMI, 66 controls	83 AMI temporal study	—	RNU6B	Creemers et al. (52)
	Serum	miR-1	8 AMI, 8 controls rats temporal study	12 AMI, 12 controls rats	31 AMI, 20 controls humans	Blood volume	Creemers et al. (52)
	Plasma	miR-122, -375	6 ST-segment elevation MI (STEMI), 6 controls	25 STEMI, 17 controls	8 STEMI temporal study	Self-normalization	D'Alessandra et al. (122)
	Plasma	miR-208b, -499	36 AMI, 36 controls	14 Acute viral myocarditis (VM), 20 post VM, 20 controls	33 Acute heart failure, 20 controls	*C. elegans* miR-39	Creemers et al. (52)
	Plasma	miR-208a	4 Controls	33 AMI, 16 coronary heart disease, 17 different cardiovascular diseases, 30 controls	5 AMI before and after treatment	Raw data	Wang et al. (123)
	Plasma	miR-499	14 Acute coronary syndromes, 15 congestive heart failure, 10 controls	—	—	Synthetic small RNA	Creemers et al. (52)
	Urine	miR-1	8 AMI, 8 controls rats	8 AMI, 8 controls rats, temporal study, serum, and urine	20 AMI, 20 controls humans	Standard curve calibration	Cheng et al. (53)
Acute kidney injury (AKI)	Plasma	miR-210	Pooled 5 AKI, 5 controls	77 AKI, 18 AMI, 30 controls	—	*C. elegans* miR-54	Lorenzen et al. (34)
	Urine	miR-21, -200c, - 423, -4640	Pooled 6 AKI, 6 controls	6 AKI and 6 healthy controls	98 AKI, 97 controls	miR-1287	Ramachandran et al. (36)
Chronic kidney disease (CKD)	Plasma and Urine	miR-16, -155, -210, -638	33 CKD, 22 controls - plasma	12 CKD, 7 controls - urine	—	Raw data	Neal et al. (20)
Acute allograft rejection	Urine	miR-10a, -10b, -210	Pooled 5 rejection, 5 stable transplant	68 Rejection, 20 stable transplant, 13 UTI after transplant	19 Before and after rejection	*C. elegans* miR-39	Lorenzen et al. (54)
Chronic allograft dysfunction (CAD)	Urine	miR-142–3p, -204, -211	13 CAD, 5 controls tissue	7 CAD, 7 controls	36 Transplant recipients temporal study	RNU48	Scian et al. (124)
ESRD	Plasma	miR-638, -21, -155, -210, -16	20 ESRD, 33 CKD, 22 controls	—	—	Raw data	Neal et al. (20)

Disease	Body fluid	Candidate miRNAs	Cohorts used			Normalizer	Reference
			Discovery	Confirmation	Evaluation		
Acetaminophen (APAP)-induced acute liver injury (ALI)	Serum	miR-122	6 APAP–no ALI, 11 no APAP–ALI, 22 CKD, 53 APAP–ALI, 25 controls,	53 APAP–ALI prospective study	11 APAP–ALI temporal study, 3 APAP–ALI before and after transplant	RNU6B	Starkey Lewis et al. (56)
Liver fibrosis	Serum	miR-29a	5 Fibrotic, 5 control mouse tissue	11 Fibrotic, 9 controls human tissue	67 Fibrosis, 17 controls	RNU6B	Roderburg et al. (125)
Chronic hepatitis C (CHC) and nonalcoholic fatty liver disease (NAFLD)	Serum	miR-34a, -122, -16	18 CHC, 19 controls	35 CHC	34 NAFLD, 19 controls	*C. elegans* miR- 238	Cermelli et al. (57)
Active pulmonary tuberculosis (TB)	Sputum	miR-3179, -147, 19b-2*	Pooled 58 TB, 32 controls	30 TB, 30 controls	-	RNU6B	Yi et al. (126)
	Serum	miR-29a	Pooled 30 TB, 30 controls	30 TB, 30 controls	30 TB; 30 controls, sputum	RNU6B	Fu et al. (127)
Biliary atresia (BA)	Serum	miR-200b, -429	5 BA, 5 controls	24 BA, 24 controls	—	*C.el*-miR-54, -238	Zahm et al. (128)

[a] List of 11 different types of organ damages in which microRNAs have been evaluated as biomarkers. Each row describes the detection matrix, study design, and normalization method used. Discovery and confirmation steps were typically performed in a cohort with a smaller sample size to select/verify candidate miRNAs, and evaluation was conducted in an expanded cross-sectional or longitudinal cohort to test the specificity, sensitivity, or early diagnostic capability. Studies were conducted using human biospecimens unless otherwise stated.

Table 4

Extracellular microRNAs as biomarkers of disease states.[a]

Disease	Body fluid	Candidate miRNAs	Cohorts used			Normalizer	Reference
			Discovery	Confirmation	Evaluation		
Sepsis	Plasma	miR-150	8 Sepsis, 8 control leukocytes	10 Sepsis, 12 control leukocytes	24 Sepsis, 32 controls	RNU6B, leukocytes; miR-192, plasma	Vasilescu et al. (59)
	Serum	miR-15a, -16, -223, -499– 5p, -122, -193b*	166 Sepsis, 32 systemic inflammatory response syndrome (SIRS), 24 controls	43 Mild, 123 severe sepsis, 24 controls	—	RNU6B	Wang et al. (129)
	Serum	miR-146a, -223	50 Sepsis, 30 SIRS, 20 controls	—	—	miR-295	Wang et al. (60)
Systemic lupus erythematosus (SLE)	Serum and urine	miR-200a, -200b, -141, -200c, -429, -205, -192	40 SLE, 30 controls– serum	40 SLE, 30 controls– urine	—	RNU48	Wang et al. (130)
	Urine	miR-146a, -155	40 SLE, 13 controls	40 SLE at time 0, 3 and 6 months after treatment	—	RNU48	Wang et al. (131)
Diabetes mellitus type 2 (DM)	Plasma	miR-126, -15a, -29b, -223, -28–3p	Pooled 2 DM, 6 controls	99 DM, 99 controls	822 Different samples	miR-454, RNU6B	Zampetaki et al. (58)
Hypertension (Hyp)	Plasma	Human cytomegalovirus miR-UL112, miR-296– 5p, let7e	13 Hyp, 5 controls	24 Hyp, 22 controls	194 Hyp, 67 controls	RNU6B	Li et al. (132)
Pulmonary arterial hypertension (PAH)	Plasma	miR-150	8 PAH, 8 controls	145 PAH, 10 controls	30 PAH	*C. elegans* miR-39	Rhodes et al. (133)
Multiple sclerosis (MS)	CSF	miR-181c, -633, -922	Pooled 10 MS, 10 other neurological diseases	53 MS, 39 other neurological diseases	—	*C. elegans* miR-39	Haghikia et al. (61)
	Plasma	miR-92a-1*, -454, -30e, -22, -210, -574–3p, -135a, -140–3p, let-7a	9 Secondary progressive MS (SPMS), 10 RRMS, 9 controls	51 SPMS, 50 relapsing remitting MS (RRMS), 32 controls	15 Amyotrophic lateral sclerosis	RNA amount	Gandhi et al. (134)
HIV encephalitis (HIVE)	CSF	miR-1224–3p, -204, -484, -720, -934, -937	5 HIV^+, 4 HIVE, 10 HIV^-	—	—	miR-622, -1266	Pacifici et al. (62)
Pregnancy	Serum	miR-520d-5p, -526a, -527	20 Pregnant temporal study, 10 controls	—	—	Panel of 6 miRNAs	Gilad et al. (135)
Bipolar disorder	Plasma	miR-134	21 Bipolar disorder temporal study, 21 controls	—	—	*C. elegans* lin-4	Rong et al. (63)

Disease	Body fluid	Candidate miRNAs	Cohorts used			Normalizer	Reference
			Discovery	Confirmation	Evaluation		
Mild cognitive impairment (MCI)	Plasma	Panel of 8 miRNAs	10 MCI, 10 controls	20 MCI, 20 Alzheimer disease, 20 controls	20 young and 20 old controls; 19 retrospective	Ratio of miRNA pairs	Sheinerman et al. (136)

[a]List of 10 different disease conditions in which microRNAs have been evaluated as biomarkers. Each row describes the detection matrix, study design, and normalization method used. Discovery and confirmation steps were typically performed in a cohort with a smaller sample size to select/verify candidate miRNAs, and evaluation was conducted in an expanded cross-sectional or longitudinal cohort to test the specificity, sensitivity, or early diagnostic capability.

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