Review

Biomarkers in Medicine

Translating extracellular microRNA into clinical biomarkers for drug-induced toxicity: from high-throughput profiling to validation

Over the past 5 years, extracellular microRNAs (miRNAs) are being vigorously explored as injury biomarkers, including drug-induced cardiotoxicity, hepatotoxicity and nephrotoxicity. Currently, the development of miRNAs as clinical biomarkers has been hindered by the lack of standardization. Therefore, extracellular miRNA-based biomarkers have not been embraced as diagnostic tools. Each platform has its strengths and weaknesses when working with low-input-amount RNA samples from body fluids; the selection of a miRNA quantification approach should be based on the study design. The following review provides a summary of the extracellular miRNA release and stability in body fluids, performances of different miRNA quantification platforms, existing clinical gold standards for drug-induced tissue damage and translation of the miRNA biomarkers from the nonclinical to clinical setting.

Keywords: biomarkers • cardiotoxicity • extracellular • hepatotoxicity • microRNA • nephrotoxicity

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MicroRNAs (miRNAs) are small non-coding RNAs approximately 22 bp in length, that regulate gene expression post-transcriptionally by binding and inhibiting particular mRNA targets. Currently, more than 1800 miRNAs have been identified and it appears that >60% of human protein-coding genes are regulated by miRNAs [1]. They typically downregulate gene expression by targeting mRNA degradation or interfering with protein synthesis process via base-pairing with complementary sequences within mRNA [2,3]. In the early 1990s, the first miRNA, lin-4, was identified in *Caenorhabditis elegans* [4]. However, it was not until 10 years later that miRNAs were recognized as unique biological regulators. During the past decade, we have learned much about the basic mechanisms of miRNA biogenesis and involvement in normal cellular function. However, more recently it has become apparent that dysregulation of miRNA has been associated with human disease. In 2008, novel reports demonstrating the presence of miRNAs in body fluids were published [5,6], since then a large number of studies have been conducted to explore miRNAs as sensitive and specific biomarkers for disease and toxicity. Several publicly available databases, miR2Disease [7] and the Human microRNA Disease Database (HMDD) [8], have been created and are valuable resources for investigating the association between miRNA and human disease.

It has been reported that miRNAs are expressed in all mammalian cells and are well conserved among species, ranging from worms to humans. In animals, miRNA have been shown to play essential roles in cellular activities, such as development, cellular differentiation, proliferation, apoptosis, cell-cycle control, metabolism and cancer. Similar to mRNA, some miRNA expression is controlled in cell- or tissue-specific manners. For instance, miR-122 is one of the dominate miRNAs in hepatocytes and variants of miR-122 accounted for approximately 72% of the total liver miRNA population [9]. To gain insights into tissue-specific miRNA distribution, we used small RNA sequencing to

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(A) Heart-enriched miRNA

miRNA	Bone marrow	Brain	Fetal Liver	Liver	Kidney	Lung	Skeletal muscle	Heart	Spleen	Thyroids
hsa-miR-197-5p	0	0	0	0	0	0	0	190.476	0	0
hsa-miR-23b-5p	0	0	0	0	0	0	0	190.476	0	0
hsa-miR-4461	0	0	0	0	0	0	0	190.476	0	0
hsa-miR-490-3p	0	569.564	291.773	174.612	0	545.455	0	67476.2	0	0
hsa-miR-1287	0	91.5882	51.9991	69.8446	94.4287	227.273	0	1380.95	0	160
hsa-miR-1	218.1818	120.21	0	69.8446	0	0	8000	1285.71	0	0
hsa-miR-133a	1672.7273	1906.18	167.553	1004.02	2696.46	15136.4	19477583.33	2861524	4200	82000
hsa-miR-208b	0	37.2077	0	0	0	0	500	3190.48	0	0
hsa-miR-499a-5p	0	297.662	43.3326	261.917	0	0	916.6667	1190.48	0	0
hsa-miR-378g	0	0	0	52.3834	0	0	833.3333	1000	0	0

(B) Liver-enriched miRNA

miRNA	Bone marrow	Brain	Fetal Liver	Liver	Kidney	Lung	Skeletal muscle	Heart	Spleen	Thyroids
hsa-miR-1295b-5p	0.00	0.00	14.44	200.80	0.00	0.00	0.00	0.00	0.00	0.00
hsa-miR-548ah-3p	0.00	20.03	17.33	680.98	0.00	0.00	0.00	0.00	0.00	0.00
hsa-miR-122-3p	0.00	0.00	1773.75	7429.72	0.00	0.00	0.00	0.00	0.00	0.00
hsa-miR-122-5p	890.91	123.07	1818367.23	5948620.57	839.37	1272.73	5083.33	3000.00	2400.00	1720.00
hsa-miR-548b-5p	0.00	0.00	0.00	165.88	0.00	0.00	0.00	0.00	0.00	0.00
hsa-miR-4662a-5p	0.00	131.66	323.55	2470.75	41.97	0.00	0.00	0.00	0.00	0.00
hsa-miR-148a-3p	419327.27	7919.52	316252.60	2222734.42	9579.27	24681.82	3750.00	8047.62	10000.00	20880.00
hsa-miR-192-3p	0.00	0.00	268.66	1981.84	83.94	0.00	0.00	0.00	0.00	0.00
hsa-miR-885-5p	0.00	2398.47	1655.30	13253.01	0.00	0.00	3000.00	0.00	0.00	160.00
hsa-miR-148a-5p	4563.64	108.76	4289.92	22359.00	723.95	1500.00	0.00	238.10	466.67	1280.00

(C) Kidney-enriched miRNA

miRNA	Bone marrow	Brain	Fetal Liver	Liver	Kidney	Lung	Skeletal muscle	Heart	Spleen	Thyroids
hsa-miR-2116-3p	0	0	0	0	62.9525	0	0	0	0	0
hsa-miR-1269b	0	0	0	0	146.8891	0	0	0	0	00
hsa-miR-204-3p	0	11.4485	0	0	2507.607	0	0	0	0	440
hsa-miR-184	0	177.4521	1120.869	0	2255.797	0	0	0	0	0
hsa-miR-513a-5p	0	0	0	0	146.8891	0	0	0	0	0
hsa-miR-30c-2-3p	127.2727	269.0403	83.7763	759.56	10922.25	2500	2666.6667	2285.714	1333.333	2160
hsa-miR-3065-3p	72.7273	88.7261	28.8884	69.8446	283.2861	0	0	0	0	0
hsa-miR-500a-5p	72.7273	11.4485	0	0	346.2386	0	0	0	0	160
hsa-miR-30a-3p	909.0909	1863.247	447.7698	4828.008	11331.44	5545.455	6666.6667	4619.048	1400	2560
hsa-miR-500a-3p	11981.8182	1416.755	4053.039	7237.646	63760.36	30409.09	305.00	14571.43	47400	19720

Figure 1. Tissue-specific miRNAs quantified by small RNA sequencing. Total RNA from human RNA tissue panel was purchased and microRNAs were quantified by Upper Quartile normalization method using next-generation sequencing. Equal amounts of total RNA were used and the expression levels were presented for each miRNA. In order to present the tissue specificity, a conditional formatting that uses a color scale to differentiate high (red), medium (yellow) and low (green) values was used for each miRNA's expression levels among the tissues. The abundancy of miRNA within the tissue can be compared by their expression levels.

analyze the miRNA profiles in selected human tissue panel (bone marrow, brain, fetal liver, heart, kidney, liver, lung, skeletal muscle, spleen and thyroids). The top ten miRNA specifically enriched in heart, liver and kidney are listed in Figure 1, with the counts values presented for each miRNA and color coded according to each miRNA's expression levels among the tissues. Similar to previous findings [10,11], miR-122–3p and miR-122–5p levels are highly enriched in the adult liver, with several thousand-fold higher expression compared with other tissues. Some miRNAs are enriched in cardiac tissue and detected at low abundance (miR-197–5p, miR-23b-5p and miR-4461); others are detected in both skeletal muscle and heart (miR-1, miR-133a, miR-499, miR-208b and miR-378g). MiR-30c-2–3p was abundant in the kidney with approximately ten-times higher expression level than other tissues, and miR-2116–3p, miR-1269b and miR-513a-5p showed kidney-specific expression patterns. Ideally, the altered extracellular miRNA after injury should be tissue-specific, such as miR-122. However, a recent study conducted in adults with APAP hepatotoxicity suggested that the highest elevations of circulating miRNAs did not come from liver [12]. One possible reason is that these miRNAs interact with key molecules in cell death and proliferation; therefore, it is not surprising to see their expression in multiple organs. In fact, some of the altered miRNAs (e.g., miR-21) in body fluids have been reported to be associated with various diseases [6].

Drug-induced cardiovascular, liver and renal injuries are the major problems that can result in failures during multiple stages of the drug development process [13]. Adverse drug reactions, as one of the leading causes of morbidity and mortality in healthcare, constitute a major clinical concern. Because of this, the 'omics' technologies have been used to explore translational biomarkers to detect the safety liabilities reliably in animals and human [14]. A biomarker is defined as a 'characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or biological responses to a therapeutic intervention' [15,16]. Ideally, novel biomarkers can be identified that will be able to monitor early indications of tissue injury to better inform clinical and regulatory decisions. A good clinical biomarker should have the following characteristics: preferentially produced in target tissue, data can be used to bridge nonclinical and clinical species, display early change for therapeutic intervention, detected through robust analytical assays and easily accessed through body fluids such as blood or urine.

In this article, we provide an overview of current knowledge of extracellular miRNA biomarkers focusing on their turnover, stability and forms of release in body fluids. Next, the technical challenges and clinical potential of extracellular miRNAs will be discussed. At last, a review of the nonclinical and clinical findings for using extracellular miRNAs as biomarkers of drug-induced cardiotoxicity, hepatotoxicity and nephrotoxicity. Certain properties of miRNAs are considered to be critical for their clinical potential: tissue origin of the biomarker and the release mechanism should be considered and clarified; sensitive to early injury in order to outperform the traditional biomarkers; specificity and correlation with established outcome measures; baseline and interindividual variance should be characterized in supportive clinical studies. Finally, reliable, quick and cost-effective extracellular miRNA quantification assays are required to promote the translation of animal miRNA biomarkers to humans.

miRNA biogenesis, turnover & carriers in body fluids

Most miRNAs are transcribed by RNA polymerase II into primary miRNAs (pri-miRNAs), which are produced in a wide array of lengths, often several thousand nucleotides long, and as seen with mRNA, are capped at the 5′ end and polyadenylated at the 3′ end. The transformation from pri-miRNAs to mature miRNA involves multiple steps at two cellular locations: in the nucleus, the pri-miRNAs are processed by the ribonuclease III enzyme Drosha in cooperation with its RNA binding partner Pasha (also called DGCR8) to stem-loop-structured molecules called precursor miRNAs (pre-miRNAs); after exported into the cytoplasm, the pre-miRNAs are processed into approximately 22-nucleotide long mature RNA duplexes by the enzyme Dicer in cooperation with the another RNA-binding cofactor TRBP (also called loquacious, Loqs). After the cleavage, the mature miRNA duplex becomes a guide strand and a passenger strand. Although either strand of the duplex may potentially function as a mature miRNA, only one strand is incorporated into the RNA-induced silencing complex (RISC) [17]. Argonaute proteins, directly interacting with miRNAs, are key factors in the assembly and function of RISC [18].

Generally, most miRNA genes produce one dominant strand, while the opposite (star or passenger) strand is preferentially destroyed according to the 'use it or lose it' strategy. Through a large-scale study with eight *in vitro* cell lines, it was recently discovered that most miRNA have slow turnover rates, with no or minor decrease within 24 h [19]. While most detected miRNAs remain at 75% levels at 12 h after transcription inhibition, a small subset of miRNAs, mostly miRNA star strands turnover more quickly. For example, miR-21–3p (or miR-21*) had a fast turnover phase

with half-life close to 1 h. Cellular miRNA expression can be regulated by changes in transcriptional level, but post transcriptional modifications affect miRNA stability; therefore, the turnover rates of different miRNA isoforms are quite different. Using deep sequencing approaches, diverse nucleotide substitutions, additions and deletions have been detected in animal miRNAs. Interestingly, the addition of a single adenine to the 3′ end of miR-122 stabilizes the molecule [20]. Therefore, the extracellular miRNAs detected in body fluids may have diverse turnover rates.

In addition to the cellular miRNA function as a gene modulator, large numbers of miRNAs have been observed in the extracellular spaces of both healthy and diseased patients. So far, miRNA have been detected in a wide range of body fluids, including serum, plasma, urine, cerebrospinal fluid, saliva, breast milk and tears. It is known that circulating cell-free miRNAs are protected from nucleases in extracellular environments; however, synthetic miRNAs were rapidly degraded when combined with nondenatured plasma [5]. This suggests that extracellular miRNAs in body fluids are not stabilized by intrinsic chemical characteristics but by various types of carriers, such as a RNA binding protein complex or extracellular lipid vesicles. Extracellular vesicles are constantly released by almost all types of cells and are present in many body fluids such as blood, saliva and urine [21]. Based on their size, these extracellular lipid vesicles have been classified as exosomes (~100 nm in diameter) or microvesicles (1–10 μm in diameter). The constituents of lipid vesicles include proteins, DNA, mRNA, miRNA and lipids. When cells undergo stress, the extracellular lipid vesicles and their constituents change accordingly [22]. Using differential fraction approaches including ultracentrifuge, size-exclusion chromatography, filtration and immunoprecipitation, it was observed that a substantial amount of vesicle-free miRNAs were associated with Ago2 [23], which protects them from degradation. Recent studies indicate that miRNAs are actively or passively released from cells via several different processes. It is hypothesized that under normal conditions, the active release of miRNAs occurs in a regulated manner. In contrast, miRNAs may be released into the circulating system in a passive process during necrotic and/or apoptotic cell death [24]. It has been shown that miR-122 is able to shift between extracellular compartments under specific pathological conditions: increased with exosome-rich fraction in alcoholic liver disease and inflammation and as a protein-rich fraction in acetaminophen-induced liver necrosis [25]. In summary, the extracellular miRNAs detected in body fluids may have diverse turnover rates, affected by their sequence features and carriers.

Analytical platforms for miRNA quantifications

Extracellular miRNA abundance in body fluids can be measured by a number of platforms, such as quantitative real-time polymerase chain reaction (qPCR) and next-generation sequencing (NGS). These technologies have significantly contributed to the identification of new miRNAs and miRNA profiles in diseases have increased our understanding of miRNA biological functions. The isoforms of some miRNAs belong to the same family and may differ from each other by only one nucleotide, making selective measurement of each isoform difficult. NGS provides a high-throughput detection of novel miRNAs and high accuracy in distinguishing miRNA isomers. The qPCR platform provides a larger dynamic range of miRNA detection and, therefore, requires less sample volume and provides more sensitive measurements than NGS. Quantifying miRNA by qPCR can be accomplished by two common methods: relative quantification and absolute quantification. The former is based on internal controls to determine fold changes and the absolute quantification gives the exact copy number by comparison with a calibration curve. Detailed discussions of the strengths and pitfalls can be found in a previous review [26].

A variety of new assay methods have been developed and some are commercially available to measure miRNAs in body fluids. Droplet digital PCR (ddPCR) is a high-throughput system that permits PCR reactions in surfactant-stabilized droplets rather than 96-well plates [27]. It combines the nanoliter-sized droplet technology with digital PCR and enables processing of approximately 2 million reactions using conventional quantification assays. Compared with qPCR, ddPCR is able to obtain highly precise absolute quantification without external references, more tolerant to the presence of inhibitors of the amplification reaction and has greater precision and improved day-to-day reproducibility [28,29]. One recent article demonstrates the capability of using ddPCR to quantify the circulating miRNAs in serum and plasma [30]. Other innovative technologies of miRNA quantifications are high-resolution MS [31] and miRNA-derived fragment length polymorphism assay [32].

The small size of miRNAs, high similarity between miRNA family members and the low abundance of miRNAs in body fluids could lead to unacceptable false-positive and false-negative results in the identification of dysregulated miRNAs. Concerns have been raised about the comparability and accuracy of results from different assay methods. Recent analyses have revealed that the performance of these methods varies, with each platform having strengths and weaknesses. There is a critical need for standard approaches to mea-

sure, report and compare the technical performance of different miRNA quantification platforms. To avoid interassay variance, it will be important to control pre- and postanalytical variables, such as RNA isolation methods, RNA quantification protocols, threshold Cq reporting and data normalization methods. In order to achieve accurate and reproducible measurements across different assays, it will be necessary to develop a set of synthetic small RNA sequences to be used as spike-in controls. The recently published microRNA quality control (miRQC) study compared and evaluated the performance of several miRNA quantification platforms [33]. Even the platforms based on the same technology may have substantial differences in reproducibility and specificity when evaluating miRNA expression. For low-input RNA, qPCR-based platforms seem to have a better score in sensitivity accompanied by high accuracy.

Extracellular miRNAs as biomarkers of drug-induced cardiotoxicity

Drug-induced cardiotoxicity is a major problem that has accounted for the failure of drug products during preclinical or clinical phases [13]. Many cancer therapy drugs are associated with a high risk of developing cardiotoxicity. For example, doxorubicin is an effective antineoplastic drug for many cancers; however, it also shows dose-dependent cardiotoxicity in both adult and pediatric patients. In addition to anthracyclines whose efficacy is jeopardized by cumulative cardiotoxicity, toxoids (e.g., taxol), alkylating agents (e.g., cyclophosphamide) and tyrosine kinase inhibitors (e.g., sunitinib) have been associated with cardiac dysfunction. In addition to the time- and money-consuming cardiac imaging techniques to monitor heart injury, serum protein biomarkers such as phosphokinase (CK), lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) have been used to monitor heart injury. Recently, myosin light chain I (MLC-I), heart fatty acid binding protein (H-FABP), creatine kinase isoenzyme MB (CK-MB), B-type natriuretic peptide (BNP) and cardiac troponins [34] have been explored and used as biomarkers of myocardial cell injury [35]. BNP is a cardiac neurohormone released from heart ventricles in response to volume expansion and blood pressure changes [36]. Cardiac troponins I (cTnI) and T (cTnT) are part of the troponin complex and regulate cardiac muscle contraction. The utility of these proteins as clinical biomarkers of drug-induced cardiac toxicity is in progress and needs further study.

Drug-induced cardiotoxicity can be classified into two categories: structural damage with direct or indirect cellular injury, leading to ischemia; and functional damage due to cardiac ion channel interruption, leading to arrhythmia. Structural toxicity may cause the release of some cellular molecules before loss of membrane integrity and investigators have studied the release of cardiac-specific miRNAs for potential use as circulating biomarkers in animals and human. A few rodent studies have been conducted to look at extracellular miRNA expression patterns during drug-induced cardiotoxicity. In 2009, the first study reported significant increases of plasma miR-208 as potential heart-specific biomarkers to detect isoproterenol-induced myocardial injury in rats [37]. Liu *et al.* investigated the potential of miR-208a as a noninvasive biomarker for myocardial injury and it appeared to be better than cTnI in a study that looked at 24 h treatment with isoproterenol in $Sod2^{+/-}$ and C57BL/6J wild-type mice [38]. A recent study explored circulating miRNA patterns in isoproterenol or doxorubicin treated rats; plasma miR-208 seems to be a sensitive biomarker for cardiotoxicity [39].

The following discussion will detail how heart-specific or muscle-specific miRNAs are being explored as biomarkers in human cardiac diseases. As one of the early studies to detect acute myocardial infarction (AMI) [40], circulating miRNAs, especially the heart-specific ones, have been explored. All muscle-enriched miRNAs, miR-1, miR-133 and miR-499, were found to be present at higher levels in the plasma of AMI patients within 4h after the onset of infarction. As one of the cardiac-specific miRNAs, plasma miR-208a levels were increased at an early time point (1 h), while it was undetected in plasma from healthy controls, patients with renal infarction or non-AMI patients with chest pain [41]. Therefore, miR-208a seemed to be an AMI biomarker with high sensitivity and specificity. Using a ligation-induced AMI model in mice [42], the potential diagnostic roles of miR-208a and miR-499 have been positively confirmed. In mice, it was observed that tissue miR-499 decreased in the myocardial infarct zone compared with the remote zone or the sham group. However, the D'Alessandra *et al.* [40]. AMI study failed to detect elevated miR-208a, and this may be explained by the relatively short half-life of miR-208a (3–12 h after AMI induction) in the blood. A family member, miR-208b, has been investigated and demonstrated to have a 1600-fold increase in plasma of AMI patients [43]. In a recent study comparing heart- and muscle-specific circulating miRNAs with cTnI, all increased in a similar trend in advanced heart failure. With the support from a left ventricular assist device, the elevation of miRNAs reversed and dropped to detection limit within 3 months [44].

As a broadly muscle-specific (enriched in muscle, not selective in cardiac tissue) miRNA, miR-1 was detected with a 200-fold peak elevation at 6 h of onset

and stable levels for at least 24 h in AMI [45]. Ai and colleagues provide results supporting the use of circulating miR-1 as a biomarker for AMI and the increases seen in AMI patients appeared to be abrogated following medication [46]. miR-133 is another broadly muscle-specific miRNA that may serve to diagnose AMI. However, due to their expression in skeletal muscle, both miR-133 and miR-1 have been linked to other diseases and thus may not offer the desired specificity [47,48].

Plasma levels of miR-423–5p have been found to be a strong diagnostic biomarker for heart failure (HF) patients, and distinguished patients with HF from healthy controls (AUC 0.91) and from patients with non-HF dyspnea (AUC 0.83) [49]. In an independent study, the elevation of circulating miR-423–5p was confirmed from HF patients relative to an age-, gender- and ethnicity-matched control group [50]. In addition, a strong correlation ($p = 0.002$) was observed between BNP and miRNA-score based on a panel of four miRNAs (miR-423–5p, miR-320a, miR-22 and miR-92b). It is likely that the elevation of circulating miR-423–5p in HF patients is caused by its upregulation in the human failing myocardium.

In conclusion, several studies fuel the notion that circulating miRNAs may serve as sensitive and specific biomarkers for heart injury. The findings from the studies described above clearly show the potential that miR-1, miR-133a, miR-499, miR-208 and miR-423–5p hold as cardiac injury biomarkers in cardiovascular diseases. The combination of miRNAs with cTnI might be useful as diagnostic and prognostic tools for patients.

Extracellular miRNA as biomarkers of drug-induced hepatotoxicity

The liver is the main organ for the metabolism and detoxification of drugs and, therefore, is often exposed to the highest concentrations of orally consumed drugs [51]. Drug-induced liver injury (DILI) is one of the leading causes of drug attrition during development and post-marketing drug withdrawal [52]. DILI is a common adverse drug reaction seen in the clinic and can be classified into two subcategories: nonidiosyncratic (predictable) or idiosyncratic. A typical example of the former is acetaminophen (APAP or paracetamol) [53], and it occurs in a dose-dependent manner. Following a toxic dose of APAP, the conjugation pathways of the liver are overwhelmed and an increased proportion of APAP is metabolized to the high reactive metabolite N-acetyl-p-benzoquinone imine. N-acetyl-p-benzoquinone imine can bind to cysteine residues in proteins, hereafter referred as APAP protein adducts. In contrast, idiosyncratic DILI is unpredictable and can occur at a therapeutic dose. Currently, the clinically used DILI biomarkers are limited and imperfect. For example, serum levels of alanine aminotransferase (ALT) is the most commonly used biomarker of hepatocellular injury, but its elevation is not liver specific [54]. Consequently, it is not surprising that efforts continue to explore sensitive biomarkers as tools to provide diagnostic value and detect safety liabilities reliably during drug development.

Some liver-enriched miRNAs were found to be good candidates for new DILI biomarkers [55]. As one of the early studies to explore circulating miRNAs as liver injury biomarkers, Wang *et al.* [56] reported plasma increases in miRNA-122 and miRNA-192 with concurrent decreases in the liver from APAP-treated mice. In addition, the level of many plasma miRNAs inversely correlated with the level of hepatic miRNAs, suggesting that for these miRNAs, hepatic injury caused the release of the miRNAs into the circulation. Interestingly, the increases in both miR-122 and miR-192 were detected earlier than the increase of ALT (1 h post-treatment vs 3 h) in the mice. In another APAP overdosed mice study, Bala *et al.* confirmed the significant increase of miR-122 in plasma [25]. In addition, both plasma miR-122 and ALT started to increase 3 and 6 h after APAP overdose, respectively, with a larger fold change increase in miR-122 than ALT. The robust increase in miR-122 was strongly associated with ALT elevation.

Similarly, the significant increase of miR-122 has been reported in APAP-overdosed rats [57-59], with increased serum levels of miR-122, miR-192 and miR-193 at 3 h while ALT remained at baseline at this time period. At later time points, ALT and miR-122 were both increased and showed peak elevations between 12 and 24 h after APAP overdose. They returned to baseline at 3 days post-treatment in a similar pattern.

In addition to liver specific miRNAs, other types of miRNAs and their exported patterns have been investigated as liver injury biomarkers. In 2012, mouse models were used to study, APAP-induced liver injury, alcoholic liver disease and Toll-like receptor (TLR) 9+4 ligand-induced inflammatory cell-mediated liver damage. In addition to the robust increase of hepatospecific miR-122, immune-related miRNAs (miR-155, miR-146a and miR-125b) were identified and demonstrated mild increases in APAP-treated mice (500 mg/kg) [25]. The increase of these miRNAs could be the result of cell death (hepatocytes and immune cells). It is found that miR-122 and miR-155 were predominately associated with the protein fraction 6 h post dosing in the APAP model, whereas they were associated with the exosome-rich fraction in alcoholic liver disease induced in mice.

To further evaluate the use of miR-122 as a clinically relevant marker of APAP-induced hepatotoxicity, several studies have been initiated to measure circulating miRNA levels in human patients [25,60]. In one clinical study, the potential of miR-122 and -192 as DILI biomarkers was explored in APAP-induced acute liver injury patients (n = 53). It is reported that both serum miRNAs were elevated in APAP overdose patients compared with healthy controls. When each miRNA was compared against peak serum ALT, serum miR-122 showed some association with ALT levels in the APAP acute liver injury cohort, whereas miR-192 showed no correlation. Another interesting observation is that miR-122 returned to baseline quicker than ALT [60]. In addition, the increase of miR-122 at earlier time points than ALT was upheld in another human DILI cohort [61]. A recent study looked at the utility of several mechanistic biomarkers in APAP overdose patients and reported that the initial measurements of miR-122, but not ALT, at first hospital presentation significantly correlated with peak ALT during the hospital stay [62]. Among patients whose initial ALT levels were in the normal range, miR-122 increases were higher in the patients who developed liver injury later, suggesting the elevation of miR-122 taken upon first presentation at the hospital could improve patient stratification and inform clinical care.

Importantly, circulating miRNA profiles could be used as biomarkers for APAP overdose diagnosis and discrimination from ischemic hepatitis [12]. Several circulating miRNAs (miR-122–5p, -27b-3p,-21–5p, -125b-5p, -194–5p, -193a-5p and -1290) contributed to distinguish APAP overdose from another common hepatotoxic pathology, ischemic hepatitis. This study also provided data on the evolution of miRNAs during treatment with the APAP antidote, N-acetyl cysteine (NAC). A majority of the elevated miRNAs recovered quickly, but miR-1290 remained elevated for at least 2 days. In a small subset of patients with high peak levels of ALT, their miRNA profiles showed a rebound trend after stopping NAC treatment. Using the next-generation sequencing approach, a recent study [63] identified 36 miRNAs on a small number of samples (n = 6) from APAP-overdosed patients. Among them, eight miRNAs (miR-107, miR-122, miR-130a, miR-148a, miR-192, miR-22, miR-27b and miR-30a) overlapped with a previous APAP mice study [56]. Diverse isomiRs could perform differently in response to liver injury. Using a sequencing approach, it was identified that miR-23a with a 3′ end variation was increased after APAP overdose, while other forms showed no significant changes. Evaluating these miRNAs', some are enriched in liver: hsa-miR-122, hsa-miR-192, hsa-miR-483–5p, hsa-miR-194–5p and hsa-miR-210–3p. Seven APAP overdose-responsive miRNAs are highly expressed in tissues other than liver, such as muscle and pancreas. It is possible that these miRNAs are released from hepatocytes as they interact with key molecules in cell death for all tissues, or APAP overdose could cause multi-organ failure.

In our recent study [64] using a small RNA sequencing approach, eight serum miRNAs (miR-122, -375, -423–5p, -30d-5p, -125b-5p, -4732–5p, -204–5p and -574–3p) were identified with more than a twofold increase in APAP overdose pediatric patients (n = 8). Importantly, there was a strong correlation between serum miR-122 and APAP protein adducts ($p < 0.01$). Sequential measurements at different days were made, and the time-dependent change of serum miR-122 followed ALT elevation. In one APAP overdose patient, the elevated serum miR-122 levels returned to baseline before serum ALT.

Compared with blood, urinary biomarkers are less invasive and may prove to be more amenable for kinetic studies. In 2012, urinary miRNA profiles were assessed for APAP-treated and carbon tetrachloride-induced liver injury in rat models [65]. After administration of a toxic dose of APAP, 44 urinary miRNAs profiles were altered. The levels of ten common urinary miRNAs, miR-185, miR-296, miR-20b-3p, miR-484, miR-330*, miR-434, miR-433, miR-34C*, miR-291a-5p and miR-664, were increased in hepatotoxicant-treated rats. The nonhepatotoxicant, penicillin, did not induce any significant changes in these ten urinary miRNAs. To explore the translational nature of these urinary miRNAs, a recent clinical study was conducted in APAP overdose pediatric patients. Urine levels of miR-375, miR-940, miR-9–3p and miR-302a were demonstrated to be increased. In contrast to published results of blood borne miR-122, the urinary level of this biomarker was not significantly changed in APAP overdosed rat or human samples.

In summary, the extent of blood borne miR-122 increase may reflect the severity of liver injury. When compared with ALT, circulating miR-122 appears to be raised earlier, with a shorter half time of several hours. Many studies have focused on APAP as an experimental model, and future work should include additional DILI scenarios to explore their application. A group of miRNAs, rather than a single miRNA, may serve as specific and sensitive markers of liver injury.

Extracellular miRNA as biomarkers of drug-induced nephrotoxicity

Sensitive and specific biomarkers are needed to detect nephrotoxicity at earlier stages than is possible today. The kidney plays a crucial role in drug excretion and detoxification. Routinely used biomarkers are serum

creatinine and blood urea nitrogen (BUN), both assessing the functional status of the kidney and detecting specific damage. Thus, these biomarkers increase significantly only after substantial kidney injury occurs and the damage may be irreversible. This stymies drug development because of the fear of inducing such morbidity as well as the clinical management of patients. Significant progress has been seen in identifying protein biomarkers of renal damage including β-2-microglobulin, clusterin, cystatin-C, kidney injury molecule-1, albumin, urinary total protein and trefoil factor 3 in rodents [66,67]. Work is underway to see if these biomarkers can be translated to the clinic and prove useful in clinical trials and standard patient care.

Urine has been used for biomarker discovery of renal injury. It is noninvasive, directly associated with renal parenchymal changes and less complex than serum. For nonclinical assessment, many new potential miRNA biomarkers of nephrotoxicity have been identified using rodent models and compared with the traditional parameters. One translational study conducted at Vishal Vaidya's lab characterizes the miRNAs in rats with kidney injury (either induced by ischemia or gentamicin treatment) and patients with clinical diagnosis of acute kidney injury (AKI) [68]. A significant upregulation of tissue miR-21, -155 and -18a was observed in the animal models and the urinary miR-21 (AUC: 0.71; p = 0.01) and miR-155 (AUC: 0.7; p = 0.02) profiles could distinguish AKI patients from controls. As a follow-up study, miR-155 deficient mice were generated and found to be more susceptible to renal injury induced by cisplatin than wild type mice. Based on gene expression analysis, c-Fos was identified as the target of miR-155 and increased apoptotic signals were found in the knockout mice [69]. Using two miRNA profiling platforms, qRT-PCR and small RNA-seq, urinary let-7d (decrease 177.8-fold; p = 0.0084), miR-203 (decrease 5.428-fold; p = 0.0158) and miR-320 (increase 2.74-fold; p = 0.0394) were identified as potential biomarkers in gentamicin-treated rats [70].

A collaborative program was performed under the Health and Environmental Sciences Institute (HESI) Biomarkers of Nephrotoxicity Committee and interesting observations were published as a companion paper in 2014 [71,72]. Cisplatin is a highly effective chemotherapeutic agent; however, the dose-dependent proximal tubule nephrotoxicity limits the achievable dosage and treatment intervals. Both studies employed a cisplatin-induced nephrotoxicity rat model to identify urine miRNA biomarkers. However, the study design differed in terms of dose, animal strain and feeding status. In one study [71], male Wistar rats received a single dose of cisplatin (0, 1 and 3 mg/kg) and urinary miRNAs were analyzed at multiple days post treatment (3, 5, 8, 15 and 26 days). Compared with vehicle controls at each time point, a large number of miRNAs reached a maximal increase on day 5 for 3 mg/kg treatment and then decreased to baseline levels on day 26. However, in this dose group, maximal tubular necrosis was detected on day 8 and the tubular regeneration was still seen on day 26. In another study, male Sprague–Dawley (SD) rats were treated with 1, 3, 6 mg/kg cisplatin, and 25 miRNAs were increased in urine [72]. Based on the histopathology, serum BUN, serum creatinine, urine Kim-1 and urine clusterin data, there is no significant difference between fed and fast condition. Similar to the previous observation, urine miR-let-7g-5p, miR-93–5p, miR-191–5p and miR-192–5p showed an increasing trend on day 3, maximizing levels on day 5 and dropping to control levels on day 7.

In 2011, Lorenzen *et al.* characterized plasma miRNA patterns among critically ill AKI patients (n = 77), healthy controls (n = 30) and acute myocardial infarction patients (n = 18). Circulating miR-210 was significantly increased in patients with AKI and the multivariable analysis indicated that miR-210 was predictive of 1-month mortality [73]. From another clinical evaluation of urinary miRNAs from AKI (n = 98) and non-AKI (n = 97) patients, Ramachandran and colleagues reported a panel of four miRNAs (with higher miR-21, miR-200c, miR-423 and lower miR-4640) with a combined cross-validated AUC (0.91; 95% CI: 0.86–0.92) [74]. Normalization has been one of the biggest challenges in analyzing urinary miRNA data. This study reported three potential normalizers, miR-307, miR-1287 and miR-489, with miR-1287 showing the most consistent performance (mean intraplate CV: 2.5%; mean interplate CV: 3.5%).

In conclusion, several urinary miRNAs are reported to show aberrant levels in AKI patients, of which miR-21 was shared between the studies. Large-scale studies are required to evaluate value as diagnostic biomarkers in AKI.

Future perspective

From the stand point of drug-induced toxicity, miRNAs have the potential to provide broad utility at various points in the drug development process. Extracellular miRNAs are great candidates as sensitive and specific biomarkers, and we can envision cell-free miRNA applications ranging all the way from nonclinical safety screening and clinical trial management to postmarket surveillance studies. There have been major advances in the discovery of miRNA species as tissue-specific toxicity biomarkers in a variety of

Table 1. Selected clinical studies exploring extracellular miRNAs as biomarkers of tissue injury.

Tissue injury	Study cohort	miRNA	Biofluids	Normalization	Ref.
Heart	17 control; 33 MI	miR-1, miR-133a, miR-133b, and miR-499	Plasma	miR-17-5p	[40]
	30 control; 33 MI, 16 non-MI CAD, 17 other CAD	miR-208a, miR-1, miR-133a, and miR-499	Plasma	cel-miR-39 spike-in	[41]
	36 atypical chest pain; 32 MI	miR-208b, miR-499, miR-133a	Plasma	Synthetic spiked-in	[43]
	39 controls; 20 HF	miR-423-5p	Plasma	miR-1249	[49]
	30 controls; 30 HF	miR-423-5p, mR-320a, miR-22, miR-92b	Serum	Average Ct value	[50]
Liver	25 controls; 53 APAP	miR-122, miR-192	Plasma	U6 snRNA	[60]
	129 APAP	miR-122	Plasma	let-7d	[62]
	22 controls; 22 APAP	miR-122	Plasma	cel-miR-39 spike-in	[61]
	12 controls; 37 APAP	miR-122	Serum/plasma	20 stable miRNAs	[12]
	6 controls; 6 APAP	miR-122, miR-192	Serum	miR-7i	[63]
	10 controls; 10 therapeutic APAP, 8 APAP overdose	miR-122 (Serum), miR-375	Serum/urine	let-7d (serum), miR-671-3p (urine)	[64]
Kidney	30 controls; 77 AKI; 18 MI	miR-210, miR-16, miR-320	Plasma	cel-miR-54 spike-in	[73]
	25 controls; 22 AKI	miR-21, miR-155	Urine	Equal amounts of RNA	[68]
	97 non AKI; 98 AKI	miR-21, miR-200c, miR-423, miR-4640	Urine	miR-1287	[74]

AKI: Acute kidney injury; APAP: Acetaminophen; CAD: Coronary artery disease; HF: Heart failure; MI: Myocardial infarction.

clinical settings; however, some miRNAs' lack of tissue specificity raises questions as to their usefulness as specific biomarkers. In the next decade, we still need a better understanding of the biological and analytic factors that affect the quantification of extracellular miRNAs.

Currently, the development of miRNAs as clinical biomarkers has been hindered by lack of standardization and concerns about the comparability of results from different assay methods. Therefore, extracellular miRNA-based biomarkers have not yet been used as diagnostic tools in clinical applications. We expect that future work that examines the accuracy, specificity, reproducibility and robustness of these miRNAs in large multicenter clinical studies will be helpful in determining their value as biomarkers. At the same time, the ongoing process of new miRNA discovery and development of quantification methods will help us enhance our ability to improve the prediction and detection of drug induced toxicities.

We expect to see further examination of these miRNA biomarker candidates in future studies conducted with more optimized study designs. First, more frequent sampling in the early stages of injury may generate more timely data that can compare the kinetic profiles of candidate biomarkers to one another. Second, it will be recommended to match the miRNA samples to ones that provide concurrent measurement of traditional blood chemistry data as well. Finally, the inclusion of another type of organ damage control to develop tissue-specific miRNA biomarkers should be included in future study design. Whether extracellular miRNAs are better predictive molecules than existing clinical biomarkers awaits confirmation in future clinical studies (Table 1).

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Executive summary

miRNA background

- MicroRNAs (miRNAs) are small noncoding RNAs that post-transcriptionally regulate the expression of thousands of genes in both normal and disease contexts.

miRNA biogenesis, turnover & carriers in body fluids

- Extracellular miRNAs are emerging as noninvasive biomarkers for drug-induced toxicities because they offer many attractive features: stability, tissue-specificity and evolutionarily conserved sequences.

Analytical platforms for miRNA quantifications

- Technological advances have spawned a multitude of platforms for miRNA profiling, and most common techniques for miRNA quantification are based on qPCR, hybridization and next-generation sequencing.
- A variety of new assay methods have been developed to measure miRNAs in body fluids, such as droplet digital PCR (ddPCR). Currently, qPCR based platforms seem to have a better sensitivity accompanied by high accuracy.

Extracellular miRNAs as biomarkers of drug-induced cardiotoxicity

- Circulating miR-1, miR-133a, miR-499, miR-208 and miR-423–5p are promising cardiac injury biomarkers in cardiovascular diseases.

Extracellular miRNAs as biomarkers of drug-induced hepatotoxicity

- Liver-enriched miR-122 has been identified as an APAP injury biomarker in animal and clinical studies.

Extracellular miRNAs as biomarkers of drug-induced nephrotoxicity

- Several urinary miRNAs are reported to show aberrant levels in kidney injury patients, of which miR-21 was shared between the studies.

Future perspective

- Validation of candidate miRNAs in larger study cohorts and accurate measurement of low amounts of miRNA in body fluids are the main challenges for their use as clinical biomarkers.

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