

# Recent progress in the use of microRNAs as biomarkers for drug-induced toxicities in contrast to traditional biomarkers: A comparative review



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## ABSTRACT

microRNAs (miRNAs) are small non-coding RNAs with 18–25 nucleotides. They play key regulatory roles in versatile biological process including development and apoptosis, and in disease pathogenesis, for example carcinogenesis, by negatively regulating gene expression. miRNAs often exhibit characteristics suitable for biomarkers such as tissue-specific expression patterns, high stability in serum/plasma, and change in abundance in circulation immediately after toxic injury. Since the discovery of circulating miRNAs in extracellular biological fluids in 2008, there have been many reports on the use of miRNAs as biomarkers for various diseases including cancer and organ injury in humans and experimental animals. In this review article, we have summarized the utility and limitation of circulating miRNAs as safety/toxicology biomarkers for specific tissue injuries including liver, skeletal muscle, heart, retina, and pancreas, by comparing them with conventional protein biomarkers. We have also covered the discovery of miRNAs in serum/plasma and their stability, the knowledge of which is essential for understanding the kinetics of miRNA biomarkers. Since numerous studies have reported the use of these circulating miRNAs as safety biomarkers with high sensitivity and specificity, we believe that circulating miRNAs can promote pre-clinical drug development and improve the monitoring of tissue injuries in clinical pharmacotherapy.

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## 1. Introduction

The emergence of drug-induced toxicity has posed a significant challenge for drug developmental processes and clinical pharmacotherapy. The lack of safety accounts for more than half of all project failures in pre-clinical animal studies and Phase I clinical trials [1]. Next to efficacy (52%), this remains a major reason (24%) for failures of phase II and III clinical trials of new molecules [2]. Once a new drug reaches the market, drug-induced toxicity may impose drug-safety related labeling changes to the drug, such as Black-box warning, and in severe cases, drug withdrawal. A recent survey investigating the post-marketing withdrawal of 462 approved drugs because of safety events, between 1953 and 2013, reported that hepatotoxicity (18%) was the most common cause for

drug withdrawal, followed by immune-related reactions (17%), neurotoxicity (16%) and cardiotoxicity (14%) [3]. Therefore, for pharmaceutical industries, safety evaluation of new molecules is quite important during the early stages of the drug developmental process to reduce the risk of attrition at a later phase.

Biomarkers form a subcategory of biological indicators that can be measured accurately and with reproducibility [4]. More precisely, it has been defined by the National Institutes of Health Biomarkers Definitions Working Group as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” [5]. Biomarkers are of several types including disease biomarkers, predictive biomarkers, prognostic biomarkers, and safety/toxicology biomarkers [6]. The different biomarkers are used according to their purposes, and contribute to clinical practice, as well as to every stage of drug development. Apart from being used during pre-clinical drug development to indicate toxicity events upon the use of a drug, safety/toxicology biomarkers are also used to monitor toxicological outcomes during pharmacotherapy, thereby contributing to

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pharmacotherapy. Currently, biomarkers are essential in clinical practice as well as preclinical drug development, and the development of enhanced approaches is refined through basic and clinical research.

Among the candidate biomarker molecules, microRNAs (miRNAs) have been gaining attention in recent times. miRNAs are evolutionarily conserved single-stranded noncoding RNAs that are 18–25 nucleotides long. miRNA genes are initially transcribed as primary miRNA (pri-miRNA) transcripts, which undergoes sequential processing and initially produces precursor miRNAs (pre-miRNAs) and finally mature miRNAs. In the cytoplasm, miRNAs modulate gene expression post-transcriptionally through guiding RNA-induced silencing complex (RISC) [7], which targets mRNA via complementary base-pairing with the 3'-untranslated region (UTR), leading to mRNA degradation or repression of translation [8]. In 2008, several research groups first discovered the presence of miRNAs in blood plasma and serum [9–11]. Since then, miRNAs have been detected in stable forms in many body fluids including serum/plasma, cerebrospinal fluid, breast milk, semen, saliva, and seminal fluid [12]. Since miRNAs are transported actively and/or passively from the cells to the extracellular space, depending upon the physiological conditions, the quantity of a specific miRNA in fluid specimens can serve as a potential noninvasive diagnostic, prognostic, and safety biomarker in many diseases such as cancers [13] and organ injuries [14].

In this review article, we have discussed the discovery of miRNAs in serum/plasma and their stability, the knowledge of which is essential for understanding the kinetics of circulating miRNA biomarkers. Subsequently, we have elaborated on the utility and limitations of circulating miRNAs as safety/toxicology biomarkers of tissue injuries frequently observed in preclinical animal studies during drug development. To adhere to the scope of the review, we have not covered the methodological challenges like sample collection, method of extraction, and normalization. However, these challenges must be overcome to enable the future application of miRNA biomarkers in humans.

## 2. miRNA biogenesis and stability

The discovery of miRNA was first reported in 1993 in lin-4, a 22-nucleotide RNA found in *Caenorhabditis elegans*, which negatively regulates the LIN-14 protein by binding to the 3'-UTR of its mRNA [15]. Since then, thousands of miRNAs have been discovered in plant, animal, and viral genomes. To date, 2654 kinds of mature miRNAs have been identified in humans, 912 in rhesus monkeys, 455 in dogs, 1978 in mice, and 764 in rats. These miRNAs have been registered in the miRBase (<http://www.mirbase.org/>) (Table 1). Recently, miRNA biogenesis or processing has been studied extensively. It involves a series of post-transcriptional processing reactions that give rise to mature miRNAs in the cytoplasm where it forms the miRNA-RISC complex which negatively regulates gene expression. On the other hand, the miRNAs are released into the extracellular space in the protein binding or vesicle-embedded form, based on the physiological condition. With increasing awareness on the use of circulating miRNAs as biomarkers for diseases, it is important to know the stability of miRNAs in the cells/tissues and blood of the body, and in separated serum/plasma. The factors affecting miRNA stability are summarized in the following sections.

### 2.1. miRNA biogenesis

The biogenesis of miRNAs has been extensively studied by many researchers. miRNAs are initially transcribed from the genome by RNA polymerase II either along with the host gene (intragenic

miRNAs), or independent of the host gene using their own promoter (intergenic miRNAs). The primary miRNA transcripts (pri-miRNA) are then recognized and cleaved by a microprocessor complex that contains RNase III Drosha [16] and the double-strand RNA binding protein, DiGeorge syndrome chromosomal region 8 (DGC8) [17]. This results in the generation of precursor miRNAs with a stem-loop structure of approximately 60–80 nucleotides in length. The pre-miRNAs in the nucleus are then exported to the cytoplasm by Exportin 5 in a Ran-GTP-dependent manner [18]. However, studies in an XPO5 (gene encoding Exportin 5) knockout human cell line showed that most miRNAs are only modestly reduced (median reduction: 0.23-fold) in abundance, indicating that Exportin 5 is not solely responsible for miRNA exportation [19]. In the cytoplasm, the pre-miRNAs are further cleaved by RNase III Dicer to produce a short (~22 nucleotides) double-stranded RNA strand (miRNA duplex) [20]. This reaction is modulated by the TAR RNA-binding protein (TARBP) and the protein activator of the interferon-induced protein kinase (PACT) [21]. Subsequently, the miRNA duplex is loaded as a double strand onto the RISC, which contains an Argonaute protein core [7]. Within Argonaute, the double-stranded RNA is unwound and the two strands dissociate from each other. One RNA strand (passenger strand) is discarded and immediately degraded by nucleases, whereas the other strand (guide strand) is retained in Argonaute and stabilized [22]. Such asymmetric strand selection onto RISC is independent of the original strand orientation, but depends on the relative thermodynamic stability of each strand and the 5' nucleotide identity [23]. Due to asymmetric strand selection, mature miRNAs originating from the 5' and 3' strands of pre-miRNA show large differences in their abundance in cells.

Considering the nomenclature of mature miRNAs, the suffix terms “-5p” and “-3p” are added and indicated as miR-X-5p and miR-X-3p, respectively, to denote the originating position of the strand on the 5' and 3' arms in the pre-miR-X hairpin. The detailed nomenclature is defined in miRBase (<http://www.mirbase.org/help/nomenclature.shtml>).

### 2.2. Stability of miRNAs within cells

miRNAs have been considered as one of the most stable forms in the RNA family [24]. A study using embryonic fibroblast from inducible *dicer1*-knockout mouse reported that the average half-life of mature miRNAs in the cells was about 119 h in comparison to that of mRNAs, which was 10 h [25]. In mouse liver, pri-mir-122 is expressed in a circadian-dependent manner with an approximate 10-fold increase in abundance during the day, whereas the expression level of mature miR-122-5p remained nearly constant over the day [26]. The absence of circadian-dependent expression in mature miR-122-5p is due to the high stability of mature miRNAs. However, it has been reported that the stability of miRNAs varies depending on the developmental time [27], tissue growth [28], and certain physiological conditions [29]. For example, the retina-specific miR-96, miR-182, and miR-204 in the mouse retina rapidly degrade within 2 h, during dark adaptation [29].

Several factors including, RNA-binding proteins and nucleotide modifications, have been reported to contribute to miRNA stability. It has been proposed that two evolutionarily conserved domains of the Argonaute protein bind to both ends of the miRNA and shield them from nuclelease-dependent degradation [30,31]. Furthermore, knockdown of the GW182 protein, a component of RISC that interacts with the Argonaute protein, reduces the half-life of miRNAs in HEK293 cells through their degradation, which is primarily brought about by the Exosome complex component RRP41 (RRP41)-dependent 3' - 5'-exoribonuclease complex [32].

**Table 1**

List of comprehensive studies on the tissue-specific expression of mature miRNAs in mammals.

Species common name (org code)	Number of miRNAs*	Method	Number of tissues	Number of specimens	Database name	Reference
Human (hsa)	2654	Microarray	24 or 37	2 males	TissueAtlas	Ludwig et al., 2016 [61]
		RNA-seq	13	1–26	HMED	Gong et al., 2014 [63]
		RNA-seq	8	1–10	miRmine	Panwar et al., 2017 [62]
		RNA-seq	41	1–12	DASHR v2.0	Kuksa et al., 2019 [64]
Cynomolgus monkey (mcf)	–	RNA-seq	27	2 males; 2 females	Iguchi et al., 2017 [60]	–
Rhesus monkey (mcc)	912					
Dog (cfa)	455	RNA-seq	16	5 males (beagle)		Koenig et al., 2016 [59]
Mouse (mmu)	1978	RNA-seq	11 or 10	4 males; 10 females		Isakova et al., preprint [58]
Rat (rno)	764	Microarray	55	3 males		Minami et al., 2014 [56]
		RNA-seq	21 or 23	5 males; 5 females	RATEmiRs	Smith et al., 2016 [57]; Bushel et al., 2018 [55]

cfa, *Canis familiaris*; hsa, *Homo sapiens*; mcc, *Macaca mulatta*; mcf, *Macaca fascicularis*; mmu, *Mus musculus*; rno, *Rattus norvegicus*; RNA-seq, RNA sequencing.\*from miRBase version 22.1 (URL: <http://www.mirbase.org/>).DASHR v2.0, Database of small human non-coding RNAs (<http://dashr2.lisanwanglab.org/index.php>).HMED, Human Micro RNA Expression Database v2.0 (<http://bioinfo.life.hust.edu.cn/smallRNA/>).miRmine (<http://guanlab.ccmb.med.umich.edu/mirmine>).RATEmiRs (<https://connect.niehs.nih.gov/ratemirs/>).TissueAtlas (<https://ccb-web.cs.uni-saarland.de/tissueatlas/>).

3'-Adenylation of miRNAs has been shown to affect their stability. The cytoplasmic poly(A) polymerase, germline development 2 (GLD2), adds a single adenosine residue to the 3' end of miR-122-5p, resulting in the stabilization of miR-122-5p in the mouse liver [33]. However, only half of the miRNAs investigated were stabilized by GLD2-mediated monoadenylation in human fibroblasts [34], suggesting that not all miRNAs are stabilized by 3'-monoadenylation. In addition to adenylation, methylation of the 2'-hydroxyl group at the 3' terminal ribose by Hen1 methyltransferase has also been shown to increase the miRNA stability in *Drosophila* [35]. Recently, 2'-O-methylation of the 3' terminal ribose, mediated by HEN1 methyltransferase homolog 1 (HENMT1), has been seen in various miRNAs in human lung cancer tissues. This modification also stabilizes miR-21-5p in a human cancer cell line, through the inhibition of cleavage by 3' - 5' exoribonucleases and by increasing the affinity for Argonaute2 [36].

### 2.3. Stability of miRNAs in separated plasma or serum

RNA molecules are considered to be unstable in plasma or serum because blood contains ribonuclease (RNase) enzymes [37]. Therefore, naked exogenous miRNAs are unstable and rapidly degrade within a few minutes in plasma [10]. However, several research groups, in 2008, have found that serum or plasma miRNAs are remarkably stable [9–11]. Later, miRNAs were also found in other body fluids including breast milk, cerebrospinal fluid, saliva, and urine [12]. miRNAs in separated plasma or serum are highly stable for 24 h at room temperature, and resistant to repeated freeze-thaw cycles and harsh low and high pH conditions [9,10]. Furthermore, serum miRNAs are stable for years at –80 °C [38]. The high stability of serum or plasma miRNAs quite contrasts that of mRNAs, which are very unstable in serum or plasma [9]. Despite the existence of studies reporting the high stability of miRNAs in serum/plasma, some reports have pointed out a decrease in the serum miRNA levels after a single freeze/thaw cycle every day for 10 days (an increase in cycle threshold (Ct) value of ~2) or an increase in the incubation at room temperature for 10 days (an increase in Ct of ~2–3) [38]. Furthermore, miRNAs in rat plasma are unstable at 37 °C or room temperature and degrade within 24 h, whereas those in human plasma are stable up to 24 h [39], suggesting the existence of differences among species in the stability of miRNAs in separated plasma/serum.

Accumulating evidence demonstrates that circulating miRNAs are encapsulated in membrane vesicles including exosomes [40],

microvesicles [41,42], and apoptotic bodies [43]. They are found to be associated with RNA-binding proteins, including Argonaute proteins [44,45] and high-density lipoproteins (HDL) [46]. However, miRNAs associated with HDL account only for a small percentage of the total circulating miRNAs [47] in human plasma or serum. Through these mechanisms, circulating miRNAs are resistant to RNase-dependent RNA degradation. However, two conflicting forms of circulating miRNAs are still a matter of debate. Two independent groups reported that the majority (>97%) of circulating miRNAs are vesicle-free and are bound to Argonaute2 in human plasma or serum [44,45]. In sharp contrast, another group demonstrated that majority (83–99%) of the miRNAs are detected in the exosome fraction of human serum [48]. Currently, the reason for this discrepancy remains unknown.

### 2.4. Stability of miRNAs in circulating blood

While the miRNAs in separated serum/plasma have been reported to be highly stable, a few studies have reported on the stability of miRNAs in circulating blood in the living body. A study employing the intravenous injection of exosomes loaded with synthetic miR-155 into *mir-155* knockout mice revealed that the half-life of miR-155 in plasma was only 5 min [49]. The same authors also reported that the intravenous injection of wild-type plasma, which contains both exosomal and protein-bound miR-155, into *mir-155* knockout mice, revealed that the half-life of miR-155 in plasma was 30 min, suggesting that miRNAs in exosomes are more rapidly cleared from circulation compared to the protein-bound forms although direct evidence for this conclusion has not been reported. It was reported that intravenously administered exosomes were taken up by the macrophages in the liver and spleen and endothelial cells in the lungs of mice. However, this could be prevented by the pre-administration of clodronate liposome which depletes the macrophages in the liver and spleen, suggesting that the rapid clearance of miRNAs is associated with macrophages [50,51]. To date, neither the clearance mechanisms, nor the stability of the protein-bound miRNAs in circulation have been reported.

Caution must be taken while using miRNAs as safety biomarkers because circulating miRNAs readily degrade and are cleared from the circulation unless necrosis is ongoing. For example, the concentration of serum miR-122-5p, which is released from damaged hepatocytes, returns to baseline earlier than the level of alanine aminotransferase (ALT) [52], which has a half-life of 47 h in

circulation [53] in patients with hepatocellular liver injury. Another example is the pancreatic islet  $\beta$  cell-abundant miR-375-3p, the level of which in serum elevates at 3 and 24 h following the administration of streptozotocin in rats. However, the level returns to baseline at 48 h when the islet cells are almost completely lost [54]. Therefore, when miRNAs are used as biomarkers, scheduled blood sampling is an important factor that must be considered.

### 3. Identification of tissue-specific miRNAs

Currently, with the advancement of omics technologies, it is possible to identify novel biomarker molecules comprehensively from the genome, transcripts (mRNA, small RNAs, and long non-coding RNA), proteins, peptides, endogenous molecules. Microarray and next generation sequencing techniques are frequently used method for the analysis of miRNAs, as they allow for genome-wide analysis of miRNAs, which enables the identification of new biomarkers. To date, thousands of mature miRNAs have been identified, and some of these miRNA sequences are conserved across species (Table 1). Since an ideal biomarker should be abundant and specifically expressed in the target organ, an understanding of the tissue specificity of miRNAs is essential for the study of miRNAs as biomarkers. Using originally obtained microarray or RNA-sequencing data, or publicly deposited data, the tissue distribution of miRNAs has been reported in rats [55–57], mice [58], dogs [59], monkeys [60], and humans [61–64]. Interestingly, some of these are provided as graphical user interface databases. As shown in Table 2 [64] and 3 [55,56], human and rat miRNA distribution in organs/tissues has been determined by microarray or RNA-sequencing, and this data is available for public view. With these data, organ/tissue-specific miRNAs have been proposed and tested to exploit their use as safety/toxicology biomarkers; these examples, if not all, are listed in Table 4 and introduced in the following sections. On the other hand, the data for monkeys and dogs are unavailable for public access.

### 4. Liver injury and biomarker

Liver is the central organ for the metabolism of major nutrients, carbohydrates, proteins, and lipids, as well as the biosynthesis of plasma proteins, hormones, and bile acids. It also plays significant roles in the detoxification of both endogenous and exogenous toxic compounds by metabolic enzymes. The liver is composed of functionally and morphologically different cell types including the parenchymal cells, namely hepatocytes (60%–70% of the total liver cell population), and several non-parenchymal cells, including cholangiocytes (2%–3%), liver sinusoidal endothelial cells (LSECs) (15%–20%), Kupffer cells, stellate cells, dendritic cells, and NK cells.

The liver is susceptible to injuries caused by xenobiotic metabolism, chemical substances, virus, alcohol, and drugs. Drug-induced liver injury (DILI) accounts for approximately 60% of the cases of acute liver failure in the United States, with acetaminophen being the most frequent cause [65]. Besides, DILI is also a major concern for drug developers. According to a survey, 462 drugs were withdrawn from the worldwide market between 1953 and 2013 owing to hepatotoxicity (81 drugs; 18%), which was the most common adverse drug reaction that led to the withdrawal [3]. DILI presents as hepatitis, cholestasis, jaundice, steatosis, nodular regenerative hyperplasia, or sinusoidal obstruction syndrome [66]. The responses of each of the biomarkers of the different conditions are different depending on the type of injury. In this section, we have presented an overview of the current biomarkers of liver injury and touched on the type-specific biomarkers, with focus on hepatocellular injury, cholestasis, and sinusoidal obstruction syndromes (SOS).

#### 4.1. Conventional biomarkers for liver injury

Current preclinical and clinical assays for the evaluation of liver injury include ALT activity, aspartate aminotransferase (AST) activity, alkaline phosphatase (ALP) activity,  $\gamma$ -glutamyl transpeptidase (GGT) activity, and total bilirubin. Serum ALT and AST activities are considered as gold standard clinical biomarkers for liver injury [67]. Although these enzymes and substance are present in tissues throughout our body, an elevation in their levels most often indicates the presence of liver injury. Hepatocellular injury essentially exhibits elevated serum ALT and AST levels compared to ALP levels, whereas cholestatic injury exhibits elevated serum GGT, ALP, and total bilirubin levels compared to ALT and AST levels [68,69]. However, false positive elevation can occur due to the limited tissue specificity.

ALT activity is routinely used for detecting liver injury. The rise in serum ALT level is generally assumed to be due to leakage from damaged hepatocytes. ALT activity is high in human liver tissue; however, other organs including kidney (50% of liver activity in Units/g of wet tissue), heart, skeletal muscle, and pancreas, also possess ALT activity [70]. Therefore, an increase in the level of serum ALT activity can result from not only the liver but also from extrahepatic tissues or cells. More specifically, ALT activity is a composite of two isozymes, ALT1 and ALT2. ALT1 is expressed in the liver, skeletal muscles, kidneys, and heart of humans, whereas ALT2 is expressed in the heart and skeletal muscles [71]. The expression of ALT1 and ALT2 in rats is largely consistent with that in humans, except that rat ALT1 is additionally expressed in the small intestine and kidneys and rat ALT2 is additionally expressed in the liver [72]. Serum AST activity is considered to be a less specific biomarker for

**Table 2**

Distribution of the miRNAs proposed as safety biomarkers in human organs/tissues.

Organ/tissue	miR-													
	124-3p	208a-3p	133a-3p	133b-3p	206	122-5p	192-5p	216a-5p	216b-5p	217-5p	375-3p	96-5p	182-5p	183-5p
Brain	1416	0	37	1	1	2	676	1	0	1	25	1	55	38
Colon	2	0	295	8	2	20	88,583	0	0	1	5909	4	449	35
Heart	0	2316	19,911	1552	1	2	87	0	0	1	0	7	4	14
Kidney	17	0	1	0	39	477	61,130	7	27	55	18	3	52	43
Liver	1	0	1	0	64	396,558	62,333	0	3	3	193	0	1	15
Lung	3	0	9	1	16	434	640	2	3	7	628	42	459	349
Eye	91	0	496	8	0	0	1035	3	2	2	8	55	1553	9176
Pancreas	11	0	6	1	160	1224	34,669	52	19,517	16,019	17,165	16	29	3
Pancreas islet	0	0	0	0	1	5	18,943	5	212	753	74,735	17	31	32
Skeletal muscle	1	1	709	91	38,299	43	147	0	0	1	3	0	0	0
Stomach	0	0	572	21	0	0	10,381	0	0	0	4544	2	17	383

Values represent reads per million (RPM). Data are derived from DASHR v2.0 (<http://dashr2.lisanwanglab.org/index.php>) [64].

**Table 3**

Distribution of the miRNAs proposed as safety biomarkers in rat organs/tissues.

Organ/tissue	miR-													
	124–3p	208a-3p	133a-3p	133b-3p	206–3p	122–5p	192–5p	216a-5p	216b-5p	217–5p	375–3p	96–5p	182	183–5p
Data derived from Minami et al., 2014 [56], 75 percentile normalization														
Brain (cerebellum)	17,428	0	0	114	128	0	99	0	0	0	0	4	0	2
Colon	0	0	1	77	0	0	11,759	0	0	0	1025	467	22	115
Heart (atrium)	0	85	1920	89,845	0	0	40	0	0	0	0	0	0	0
Kidney	0	0	0	24	0	19	4310	0	0	0	27	377	21	139
Liver	1	1	1	1	1	213,182	10,296	1	1	1	118	1	1	1
Lung	0	0	2	131	0	3	41	0	0	0	253	214	8	95
Eye	11,038	0	34	1786	106	0	42	0	0	0	28	8978	366	3979
Pancreas	1	1	1	1	51	1	1	2615	282	1506	2657	1	1	1
Skeletal muscle (soleus)	4	0	2665	114,314	23,681	0	28	0	0	0	0	0	0	0
Small intestine (jejunum)	0	0	0	131	0	0	23,712	0	0	0	631	692	52	196
Stomach (anterior)	0	0	58	2550	0	7	42	0	0	0	0	231	24	88
Data derived from RATEMiR [55], trimmed mean of M values (TMM) normalization														
Brain (cerebellum)	400,953	0	3148	63	660	42	120,553	67	21	254	1872	305	34,114	5298
Heart	61	18,671	5,410,000	1609	10	90	21,861	31	2	55	268	11	1128	85
Kidney	50	2	2431	3	4	45	2,560,000	16	3	25	25,119	2150	298,712	29,456
Liver	84	3	7020	3	18	1,190,000	5,210,000	12	4	8	13,604	428	57,212	2742
Pancreas	165	4	4378	2	6	343	60,854	218,734	29,756	263,910	1,640,000	3712	317,267	14,909
Skeletal muscle (soleus)	307	13	9,710,000	15,958	70,338	70	22,988	15	3	30	197	45	9086	576
Small intestine (jejunum)	180	15	28,142	10	13	127	11,400,000	9	2	20	224,923	1035	183,511	16,486

**Table 4**

Tissue-specific expression of miRNAs and their application as circulating biomarkers for tissue/organ injuries.

Target organ	Tissue-specific miRNA	Species	Treatment	Reference
Brain	miR-124–3p	Rat	Transient/permanent ischemia	Laterza et al., 2009 [83]
Heart	miR-208a-3p	Rat	Isoproterenol, metaproterenol, allylamine	Ji et al., 2009 [142]; Calvano et al., 2016 [146]
Heart, skeletal muscle	miR-133a-3p, –133b-3p	Rat	TMPD, HMG-CoA reductase inhibitor, isoproterenol, metaproterenol, mitoxantrone	Laterza et al., 2009 [83]; Calvano et al., 2016 [146]
Skeletal muscle	miR-206a-3p	Rat	Allylamine, cerivastatin, PPAR $\alpha/\gamma$ agonist, TMPD	Bailey et al., 2019 [91]
Liver	miR-122–5p	Rat	CBrCl <sub>3</sub> , CCl <sub>4</sub>	Laterza et al., 2009 [83]
Intestine	miR-122–5p, -192–5p miR-215–5p	Mouse Rat	Acetaminophen Diclofenac, HSP90 inhibitors, PAK4 inhibitors	Wang et al., 2009 [82]; Kalabat et al., 2017 [191]
Pancreas	miR-217–5p, –216a-5p, –216b-5p, –375–3p	Rat	Caerulein, streptozotocin	Calvano et al., 2016 [54]; Erdos et al., 2020 [178]
Retina	miR-96–5p, -124–3p, –182, -183–5p	Rat	Pan-CDK inhibitors, NaO <sub>3</sub> , laser-induced choroidal neovascularization injury, MNU, NMDA	Peng et al., 2014 [166]; Peng et al., 2016 [159]; Kakiuchi et al., 2019 [165]

CDK: cyclin-dependent kinase; HSP90: heat-shock protein 90; MNU: N-methyl-N-nitrosourea; NMDA: N-methyl-D-aspartate; PAK4: p21-activated kinase 4; TMPD: 2,3,5,6-tetramethyl-p-phenylenediamine.

liver injury compared to ALT activity. AST is expressed in a broad spectrum of tissues including liver, heart, skeletal muscle, kidneys, brain, pancreas, lungs, leukocytes, and erythrocytes, in an isozyme-dependent manner [73]. Similar to ALT, a rise in the level of serum ALT activity acts as a biomarker for liver injury. However, it can also elevate in response to heart and skeletal muscle injury [74,75].

Serum ALP and GGT levels are elevated during cholestasis or biliary injury. The ALP isozymes include high molecular weight ALP (ALP1), hepatic ALP (ALP2), bone ALP (ALP3), placental ALP (ALP4), intestinal ALP (ALP5), and IgG-bound ALP (ALP6). ALP2 and ALP3 can be detected normally in the serum. GGT is expressed in the liver, kidneys, and pancreas [76]. Basically, the elevation of the serum ALP and GGT levels during biliary disease shows the same profile. However, GGT is less reliable as an ALP for detecting

cholestasis in humans [77]. Conversely, GGT activity is considered as a reliable marker for cholestasis compared to ALP in rats [67].

#### 4.2. Novel protein and miRNA biomarkers for hepatocellular injury

##### 4.2.1. Protein biomarkers for hepatocellular injury

Novel investigational proteins and miRNAs have been reported for the detection of hepatocellular injuries. Among them, serum glutamate dehydrogenase (GLDH) is the most promising. GLDH is more specifically expressed in the liver, compared to ALT and AST. Its primal distribution is in the liver, and it is distributed to a lesser extent in the kidneys (11% of enzyme activity, units/g, in the liver), brain (8%) and lungs (6%), with only trace amounts in muscle tissues [78]. In both clinical and nonclinical studies, serum GLDH has

been shown to be more sensitive than ALT, and it is unaffected by muscle diseases. In rats, compared to an enzymatic increase in plasma ALT, the elevation in GLDH was up to 10-folds greater in magnitude, and the elevation persisted up to 3-folds longer after treatment [79]. In healthy subjects, the serum GLDH levels were not affected by age or gender with reference ranges of 1–10 U/L [80]. The serum GLDH level elevated during minimal acetaminophen-induced liver injury in a mouse model of Duchenne muscular dystrophy, while the serum ALT level did not [81]. Serum GLDH levels in 131 subjects with a variety of muscular diseases were the same as those in healthy subjects while the serum ALT levels correlated with the serum creatine kinase (CK) level [81]. Based on data submitted by the Critical Path Institute's (C-Path) Predictive Safety Testing Consortium's (PSTC) Hepatotoxicity Working Group (HWG) and the Duchenne Regulatory Science Consortium (D-RSC), the European Medicines Agency (EMA), in 2017, issued a Biomarker Letter of Support for serum GLDH for monitoring hepatocellular injury. Further clinical studies will permit the potential use of GLDH as a biomarker in the future.

The first discovery of the use of miRNA biomarker for liver injury occurred a decade ago. In 2009, Wang et al. reported that the plasma levels of miR-122–5p and miR-192–5p in mice increased up to 250-fold during acute hepatocellular injury induced by acetaminophen [82]. Several subsequent studies confirmed the elevation in the levels of miR-122–5p and miR-192–5p during liver injury in rats [39,83], beagle dogs [59], cynomolgus monkeys [84], and humans [85–87]. Importantly, circulating miR-122–5p and miR-192–5p levels increased as early as 1 h after acetaminophen administration while plasma ALT level increased after 3 h [82], suggesting that miRNAs can be early predictive biomarkers of high sensitivity for hepatocellular injury. miR-122–5p is liver- and hepatocyte-specific (Fig. 1A and Fig. 2), accounting for 70% of all hepatic miRNAs in the liver, with negligible expression in the extrahepatic tissues [88] of humans (Table 2 [55]), rats (Table 3 [56]), mice [88], monkeys [60], and dogs [59]. miR-192–5p is considered liver-enriched because it is substantially expressed in the liver, kidneys, and gastrointestinal tract (Tables 2 and 3). Therefore, elevation in the level of circulating miR-192–5p does not always indicate liver injury. In fact, circulating miR-192–5p level increased up to ~10-fold during kidney injury induced by ischemic reperfusion in rats [89].

#### 4.2.2. miRNA biomarkers for hepatocellular injury

Recently, a comparative performance of circulating miR-122–5p and miR-192–5p as biomarkers for liver injury induced by various hepatotoxins has been reported in rats [90,91]. In acute and sub-acute toxicity rat studies using 23 compounds, miR-122–5p (area under the receiver operator characteristic curve, ROAUC: 0.91) outperformed ALT (ROAUC: 0.82;  $p < 0.05$  versus miR-122–5p) and was almost equal to GLDH (ROAUC: 0.89; not significant versus miR-122–5p) [90] in the diagnosis of histopathologically confirmed liver injury. In another report of 28 acute toxicity rat studies using 10 hepatotoxins and 18 non-hepatotoxins with toxicity to heart, kidneys, pancreas, or skeletal muscle tissues, miR-122–5p, miR-192–5p, and GLDH exhibited high ROAUC values  $> 0.96$  and a sensitivity  $>80\%$  at 95% specificity, while ALT (ROAUC: 0.94) and AST (ROAUC: 0.91) had a relatively poor sensitivity  $< 80\%$  at 95% specificity [91]. Therefore, miR-122–5p and GLDH are more valuable than ALT in monitoring liver injury in acute and sub-acute rat toxicity studies.

For the clinical application of circulating miR-122–5p, the upper limit of the normal range needs to be established by considering inter-individual variance. It has been reported that serum miR-122–5p shows the largest inter-individual variability (117-fold, Table 5) among the miRNAs tested in 240 healthy human subjects

in the United States [92]. In other studies, a 38- to 74-fold variance (Table 5) was observed in the serum miR-122 level in healthy subjects [87]. These inter-individual variance values of miR-122–5p in circulation are larger than those of protein biomarkers like serum ALT (5-fold) [92] and GLDH (7- to 9-fold) [87]. Therefore, the large inter-individual variance may limit the clinical utility of circulating miR-122–5p as safety biomarkers for detecting liver injury. However, lower variance in serum miR-122–5p level has been reported in experimental animals: 20-fold in 50 cynomolgus monkeys [60] and 50-fold in 154 rats [90] (Table 5). Besides, serum miR-122–5p levels outperformed ALT as a biomarker in histologically confirmed liver injury in rats [90,91], suggesting that serum miR-122–5p can be utilized as a biomarker for hepatotoxicity in acute and sub-acute rat toxicological studies.

#### 4.3. Novel miRNA biomarkers for cholestasis or bile duct injury

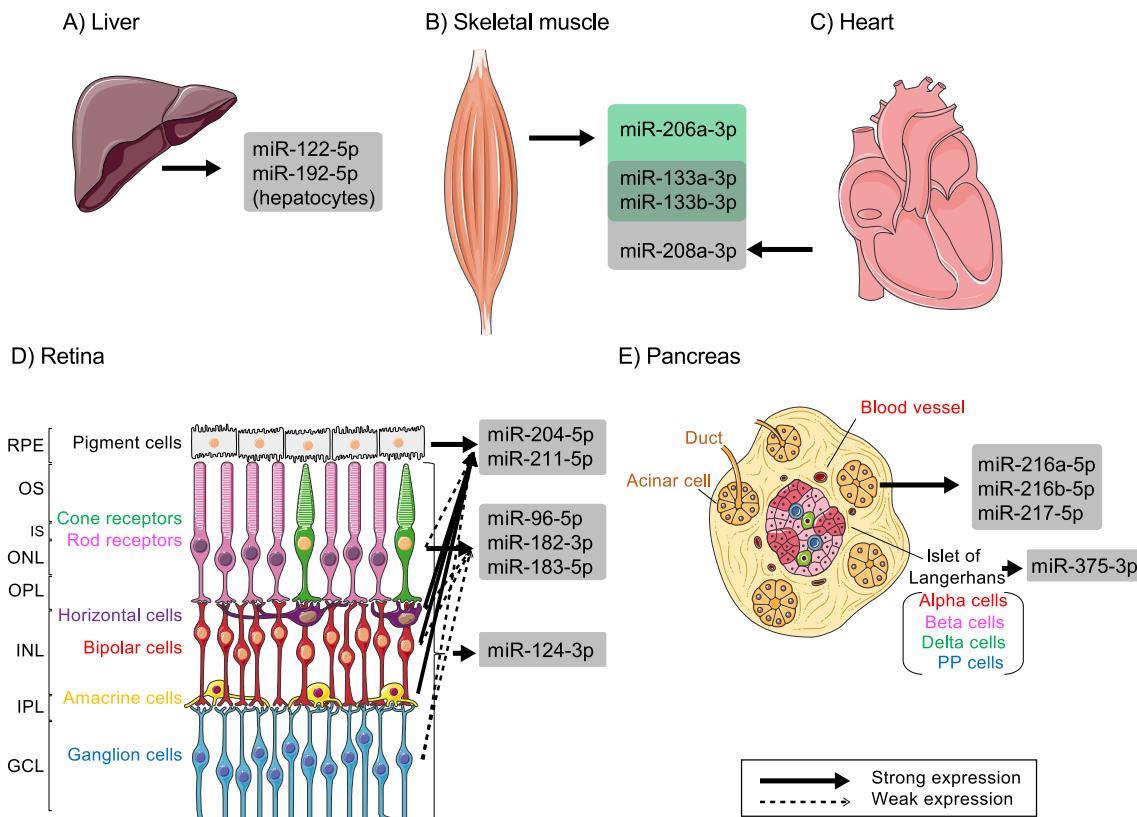
Cholestatic injury presents elevated GGT, ALP, and bilirubin levels compared to ALT and AST levels [68,69]. However, an elevation in the levels of these biomarkers does not necessarily indicate the presence of liver abnormality because tissue localization is not limited to the liver. Our group recently reported an increase in the levels of miR-143–3p and miR-218a-5p up to 30-fold in the plasma of rats with acute bile duct injury induced by  $\alpha$ -naphthylisothiocyanate and 4,4'-methylenedianiline; however, the levels of these miRNAs were not increased during hepatocellular injury induced by acetaminophen or thioacetamide or during steatosis induced by carbon tetrachloride or dexamethasone [93]. Interestingly, miR-218a-5p is expressed in rat cholangiocytes but not in hepatocytes, suggesting the leakage of miR-218a-5p into the blood during cholangiocyte injury (unpublished result). However, it should be noted that plasma miR-218a-5p levels generally return to the baseline value earlier than ALP or GGT. This may be due to the number of cholangiocytes, which account for only 3% of the liver cell population. Further validation of this finding using other cholestasis and bile duct injury models is warranted.

#### 4.4. Biomarkers for sinusoidal obstruction syndrome (SOS)

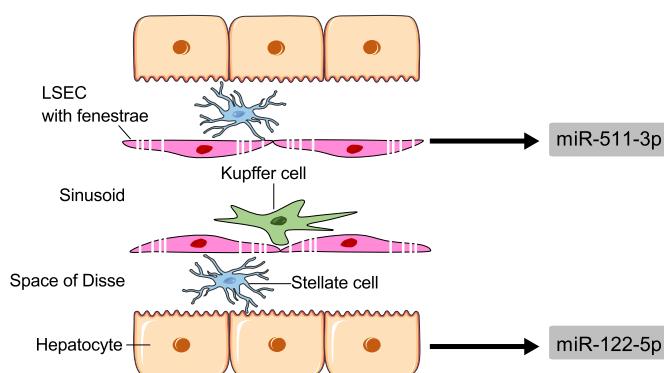
Sinusoidal obstruction syndrome (SOS) is a complication resulting from clinical irradiation and chemotherapy treatments used during hematopoietic stem cell transplantation. Drugs used in the conditioning regimen damage the LSECs, thereby leading to narrowing or fibrous obliteration of terminal hepatic venules and small lobular veins. Obstruction of blood flow due to venous occlusion leads to liver enlargement, ascites, and hepatocellular necrosis [94]. Although most cases of SOS resolve within a few weeks, the most severe forms can result in fulminant hepatic failure and multi-organ failure, with a high mortality rate  $>80\%$  [95]. Drugs that have been reported to be associated with SOS include oxaliplatin, busulfan, dacarbazine, cyclophosphamide, and gemtuzumab-ozogamicin [96]. The clinical diagnosis of SOS is done according to either the Baltimore criteria or the Seattle criteria, both of which are based on the total serum bilirubin level, painful hepatomegaly, ascites, unexplained weight gain [97,98] with no disease specificity, and late events in disease development. The incidence of SOS varies depending on the criteria used: 17.3% (Seattle) and 9.6% (Baltimore). Now that defibrotide, a therapeutic drug, has been shown to be effective for the prevention and treatment of SOS, the development of early predictive biomarkers for SOS clinical practice is important.

#### 4.4.1. Protein biomarkers for SOS

Previously, several potential biomarkers were proposed for the detection of SOS in patients based on the hypothesis that endothelial damage precedes the occurrence of SOS. These include



**Fig. 1.** Tissue-specific miRNAs in (A) liver, (B) skeletal muscle, (C) heart, (D) retina, (E) pancreas of mammals. This figure was created in part using images from Smart Servier Medical Art (<https://smart.servier.com/>), which is licensed under a Creative Commons Attribution 3.0 Unported License (<https://creativecommons.org/licenses/by/3.0/>). GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, outer segment; RPE, retinal pigment epithelium.



**Fig. 2.** Schematic structure of the liver and specific miRNAs in hepatocytes and liver sinusoidal endothelial cells (LSECs) in rats.

circulating angiopoietin-2 (ANG2), E-selectin, hyaluronic acid (HA), intercellular adhesion molecule-1 (ICAM-1), plasminogen activator inhibitor-1 (PAI-1), suppression of tumorigenicity-2 (ST2), thrombomodulin, vascular endothelial growth factor (VEGF), and von Willebrand Factor (vWF) [99–103]. However, comparison of the prediction performance of these biomarkers has not been performed thus far. One of the more recent studies by Akil et al. employed a quantitative mass spectrometry-based proteomics approach and identified 6 candidate proteins among 494 proteins in 20 patients with SOS and 20 patients without SOS. These include

L-Ficolin, vascular cell adhesion molecule-1 (VCAM1), tissue inhibitor of metalloproteinase-1 (TIMP1), vWF, ICAM1, and CD97 [104]. They further found that ST2, ANG2, L-Ficolin, HA, and VCAM1 exhibited a higher diagnostic performance for SOS among the 6 proteins and 5 additional markers [ST2, ANG2, HA, thrombomodulin, and PAI-1] selected from previous reports. L-Ficolin, HA, and VCAM1 also stratified patients at risk for SOS as early as the day of hematopoietic stem cell transplantation, suggesting that these 3 biomarkers enable early identification and intervention to minimize SOS incidence.

#### 4.4.2. miRNA biomarkers for SOS

We sought to identify miRNAs which could serve as SOS biomarkers in rats [105,106]. From the microarray analyses of miRNAs obtained from the isolated rat LSECs and hepatocytes, and the organ specificity data of miRNAs, miR-511-3p was found to be abundant in LSECs and relatively enriched in the liver (Fig. 2). In rats treated with monocrotaline, which induces SOS-like phenotypes in rats [107], the level of circulating miR-511-3p increased after 6 h of monocrotaline administration, while the level of miR-122-5p did not get altered during that time, suggesting the release of miR-511-3p from the damaged LSECs into the circulation. Therefore, miR-511-3p can serve as a biomarker for SOS. However, it should be noted that the sequence of miR-511-3p in rats is one nucleotide shorter than that in humans and the organ specificity of miR-511-3p in humans remains poorly understood [105]. An investigational study is required to identify the circulating miRNAs which can serve as biomarkers for the detection of SOS in patients.

**Table 5**

Variability of tissue-specific or -enriched miRNAs in circulation in healthy individuals.

miRNA	Target organ	Species	Variability in serum or plasma (fold)	Number of specimens	Normalization	Reference
miR-122-5p	Liver	Human	38; 74 <sup>a</sup>	81; 192	mmu-miR-293	Church et al., 2019 [87]
		Human	117 <sup>b</sup>	240	cel-miR-39	Vogt et al., 2019 [92]
		Monkey	20.2 <sup>c</sup>	50	cel-miR-238	Iguchi et al., 2017 [60]
		Rat	~50 <sup>d</sup>	154	cel-miR-39 and cel-miR-54	Sharapova et al., 2016 [90]
miR-192-5p	Liver	Monkey	7.9 <sup>c</sup>	50	cel-miR-238	Iguchi et al., 2017 [60]
miR-1-3p	Muscle	Monkey	581 <sup>c</sup>	50	cel-miR-238	Iguchi et al., 2017 [60]
miR-133a-3p	Muscle	Human	61.5 <sup>b</sup>	240	cel-miR-39	Vogt et al., 2019 [92]
		Monkey	971 <sup>c</sup>	50	cel-miR-238	Iguchi et al., 2017 [60]
miR-206-3p	Muscle	Monkey	426 <sup>c</sup>	50	cel-miR-238	Iguchi et al., 2017 [60]

cel, *Caenorhabditis elegans*; mmu, *Mus musculus*.

Fold differences were calculated based on the following formulas.

a: 95th percentile/5th percentile.

b: 95% confidential interval.

c: (mean + 2SD)/(mean - 2SD).

d: (mean + 2.7SD)/(mean - 2.7SD).

### 5. miRNAs as biomarkers for extrahepatic tissue toxicities

Based on the published tissue specificity data of miRNAs in humans and experimental animals, many efforts have been taken to identify, evaluate, and validate the use of miRNAs as biomarkers of extrahepatic organ toxicity. Typically, circulating miRNAs indicating heart, skeletal muscles, pancreas, and retina toxicity have been associated with the organ-specific expression of these miRNAs in more than two independent studies (Table 4). Here, we have provided a brief summary of such organ-specific miRNAs and compared currently available biomarkers. Although some studies have proposed miRNAs as biomarkers for kidney injury, they are not consistent across studies [108]. Likewise, circulating miRNAs for interstitial pneumonia, which is a serious adverse event occasionally observed during chemotherapy, have not been proposed probably due to difficulties in establishing an animal model of interstitial pneumonia.

#### 5.1. Skeletal muscle toxicity

Skeletal muscle toxicity is known to be induced by a variety of drugs, including statins, fibrates, antibacterial agents, proton pump inhibitors, and immunosuppressants in clinical pharmacotherapy [109,110]. Generally, the incidence of rhabdomyolysis, a severe skeletal muscle disorder, is not high: 0.44 per 10,000 person-years for monotherapy with statins (atorvastatin, pravastatin, or simvastatin), 2.82 for fibrates, and 5.34 for cerivastatin [111]. However, the incidence increased to 5.98 when a combination of atorvastatin, pravastatin, or simvastatin and a fibrate was used, and to 1035 when a combination of cerivastatin and a statin was used. In total, 52 deaths were reported in patients using cerivastatin, mainly due to rhabdomyolysis and the resultant renal failure [112,113]. Eventually, cerivastatin was withdrawn from the markets worldwide in 2001. Therefore, life-threatening rhabdomyolysis must be prevented by early diagnosis.

Currently, traditional biomarkers including serum AST activity and CK activity are being widely used for the detection of skeletal muscle disorders. However, they lack sensitivity and tissue specificity, partly due to their expression in extraskeletal muscle tissues, which hampers early sensitive diagnosis [114,115]. Several protein biomarkers for skeletal muscle injury have been evaluated, first in patients [116–118], and then in rats reverse-translationally [119,120]. Based on clinical evaluation, the promising muscle injury biomarkers include skeletal troponin I (sTnI), myosin light chain 3 (Myl3), fatty acid binding protein 3 (FABP3), and CK-MM [homodimer of CK subunit M (muscle type)]. sTnI is a biomarker that is exclusively expressed in the skeletal muscles. It has two

isoforms, the troponin I slow-twitch isoform, which is specific to the slow-twitch type I fiber, and the troponin I fast-twitch isoform, which is specific to the fast-twitch type II muscle fiber [119,121]. In contrast, Myl3, FABP3, and CK-MM are expressed in both skeletal muscles and heart, thereby making it impossible to discriminate heart and skeletal muscle injuries using these 3 biomarkers in circulation. The molecular characteristics of these biomarkers have been previously described in detail [91,115,122]. The first comparative study using rats treated with 6 myotoxic compounds such as allylamine, cyclosporine A, doxorubicin, isoproterenol, metaproterenol, and mitoxantrone, and 6 non-myotoxic compounds with toxicity to other tissues revealed that sTnI, FABP3, and Myl3 outperformed AST activity and CK activity in the detection of skeletal muscle injury [119]. Subsequently, the C-Path PSCT skeletal muscle working group was established to demonstrate the biological relevance and toxicological responsiveness of sTnI, Myl3, CK-MM, and FABP3 as skeletal muscle injury biomarkers in rats [122]. They employed 7 compounds with skeletal muscle toxicity and 14 compounds with toxicities to tissues other than skeletal muscles, and successfully showed that sTnI, Myl3, CK-MM, and Fabp3 were superior to AST activity and/or CK activity. Based on the eligibility criterion, the U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) issued a Letter of Support in 2015 for the use of sTnI, Myl3, Fabp3, and CK-MM as biomarkers of skeletal muscle degeneration/necrosis in preclinical development, and encouraged their use in early clinical trials [123,124].

The tissue distribution of myomiRNAs (muscle-specific miRNAs) has been reported previously. miR-206-3p is almost exclusively expressed in skeletal muscles whereas miR-133a-3p, miR-133b-3p, and miR-1-3p are expressed in both the heart and skeletal muscles of mice [125], rats (Table 3) [91,126], monkeys [60], and humans (Table 2) [127] (Fig. 1B). The mouse and human genomes contain two miR-1/133a gene clusters located on two different chromosomes, encoding identical mature miR-1-3p and miR-133a-3p, whereas the rat genome contains only one miR-1/133a gene cluster. In contrast, the miR-206/133b cluster is located on a single chromosome in humans, mice, and rats. The reason behind the expression of miR-133b-3p in both the heart and skeletal muscles and the restriction of the expression of miR-206-3p to only the skeletal muscles is not known. However, it is suggested that miR-206 and miR-133b arise from two separate primary transcripts under the control of independent promoters [128]. In mice, both miR-206 and miR133b genes are encompassed by the primary long intergenic non-coding RNA, muscle differentiation 1 (linc-MD1), which is transcribed by an upstream distal promoter. However, only pre-miR-133b is released during the maturation of the pri-linc-MD1 into mature spliced linc-MD1 [128]. In contrast, the primary

transcript of miR-206 is transcribed by a proximal promoter located in the intron of the linc-MD1 gene [128].

The use of miR-206–3p as a biomarker for skeletal muscle injury in rats was first reported in 2009 – the same time the circulating miRNA toxicity biomarkers of other organs were identified [83] (Table 4). An elevation in the level of plasma miR-206–3p and miR-133b-3p has also been observed in mice with skeletal muscle injury induced by pioglitazone [129], a combination of lovastatin and gemfibrozil [130], and a combination of ciprofloxacin and atorvastatin [131]. Recently, traditional biomarkers (AST activity and CK activity), novel protein markers for muscle injury (sTnI, Myl3, FABP3, CK-MM), and myomiRNAs (miR-206–3p, miR-133a-3p, miR-133b-3p, and miR-1-3p) were comprehensively evaluated as biomarkers for skeletal muscle injury in rats, using 27 compounds which induced injury not only to the skeletal muscles but also to the kidneys, liver, heart, pancreas, and gastrointestinal tract [91]. They reported that the myomiRNAs (ROAUC: 0.94–0.95) and novel protein markers (ROAUC: 0.88–0.94) outperformed the traditional biomarkers for the detection of skeletal muscle injury, which showed ROAUC values of 0.81 for AST, 0.76 for ALT, and 0.69 for CK. Remarkably, sensitivity values of 89.9% for miR-206–3p and 88.4% for miR-133a-3p at 95% specificity were the highest. This was followed by 88.1% for Myl3, 87% for miR-133b-3p, 85% for FABP3, 78.3% for miR-1-3p, 76.1% for CK-MM, and 70.1% for sTnI, suggesting that these myomiRNAs could add more value, with respect to sensitivity and specificity, in the detection of skeletal muscle injury, compared to the traditional biomarkers. However, in a panel of control samples, large individual variability was seen among the myo-miRNAs (ex. 426-fold for monkeys [60]) in circulation (Table 5). Therefore, individual differences in these miRNAs should be evaluated more carefully.

## 5.2. Cardiac toxicity

Drug-induced cardiotoxicity is a life-threatening adverse reaction. Analysis of pharmaceutical withdrawals from the European Union market between 2002 and 2011 reveal that cardiovascular events or disorders (9 drugs, 47% for safety reasons) were the main reason for the withdrawal of 19 drugs, removed from the market due to safety reasons [132]. Therefore, it is important for preclinical studies to identify any potential cardiotoxicity induced by candidate drugs. Meanwhile, certain drugs used in cancer therapy including anthracyclines, 5-fluorouracil, trastuzumab, as well as a number of small molecule kinase inhibitors, have been shown to be potentially associated with cardiotoxicity [133,134]. For such drugs, it is essential to have safety biomarkers to closely monitor cardiotoxicity, both during drug development and post-marketing medication. Degenerative structural effects mediated by myocardial necrotic injury can be monitored by serum biomarkers because this type of cardiotoxicity, e.g. myocardial infarction, is accompanied by the release of cell constituents [135].

Serum AST activity, lactate dehydrogenase activity, and CK activity have been used as biomarkers for cardiotoxicity. However, they lack sensitivity and specificity to detect skeletal muscle injury [136]. Current biomarkers such as CK-MB [heterodimer of CK subunit M (muscle type) and subunit B (brain type)], cardiac myoglobin, FABP, and cardiac troponins (cTn) I and T have been widely used in clinical diagnosis [137]. Among these, cTnI and cTnT are currently considered as the gold standard for the diagnosis of acute myocardial infarction in humans, as proposed by the members of a global joint task force [138]. Both cTnI and cTnT have been reverse-translationally applied in experimental animals. To date, their use as biomarkers for cardiotoxicity has been reported in rats, mice, and dogs treated with cardiotoxic drugs. These animal studies have been summarized in a previous review [135]. However,

several drawbacks need to be taken into consideration when using cTnI and cTnT as biomarkers for cardiotoxicity in experimental animals. First, in rats, cTnI and cTnT rapidly disappear from the circulation within a few hours after the cardiac event [135], whereas their levels remain elevated for days in humans [139]. Therefore, animal experiments require appropriate time points for blood sampling. Furthermore, cTnI and cTnT levels increase during chronic kidney disease and end-stage renal disease because they are excreted from the kidneys [140,141]. However, the effect of this increase in experimental animals remains unknown.

The use of circulating miRNAs as biomarkers for cardiotoxicity has been reported (Table 4). For example, miR-208a-3p is specifically expressed in the heart tissue of mice [125], rats (Table 3) [126], and monkeys [60] (Fig. 1C). First, it was reported that serum miR-208a-3p was not detectable at baseline but could be detected as early as 3 h after the administration of isoproterenol in rats, which induced acute myocardial infarction. The kinetics of isoproterenol was almost equal to that of plasma cTnI level [142]. Although some studies have focused on four miRNAs (miR-1-3p, miR-133a-3p, miR-133b-3p, and miR-499) as biomarkers for cardiac injury, these miRNAs are expressed both in the heart and skeletal muscles of both humans and rats [127,143–145] (Fig. 1C). In rats with cardiac injury induced by a single dose of isoproterenol administered intravenously, the level of plasma miR-208a-3p consistently increased for 24 h, whereas that of cTnI and cTnT transiently elevated and returned to baseline at 24 h [126]. Similarly, rat models with cardiac injury induced by a single dose of isoproterenol (s.c.), allylamine (p.o.), and metaproterenol (i.p.), showed a constant 2- to 6-fold increase in the level of serum miR-208a-3p at 4, 24, and 48 h post administration. However, the level of serum cTnI increased at 4 h post metaproterenol administration and then decreased; the administration of the other cardiotoxic compounds did not cause an increase in cTnI level [146]. Other researchers reported that, similar to cTnI and cTnT, the level of plasma miR-208a-3p transiently increased and returned to normal by 24 h post administration of isoproterenol (i.p.) in rats [147]. Collectively, it would be advantageous to use heart-specific miR-208a-3p as a circulatory biomarker for cardiotoxicity in rats, owing to its relatively constant level after injury.

## 5.3. Retinal toxicity

Drug-induced ocular toxicity is presented as keratitis (cornea), retinopathy (retina), cataract (lens), and glaucoma. Pfizer researchers reported that ocular toxicity accounts for approximately 7% of attrition of internal therapeutic candidates, the 4th major reason of attrition after cardiovascular, liver, and kidney toxicity. In addition, 99% of the ocular toxicity was attributed to retinal toxicity [148]. In clinical pharmacotherapy, many systemic drugs including, chlorpromazine, chloroquine, hydroxychloroquine, sildenafil, tamoxifen, and vigabatrin cause retinopathy [149,150]. For functional retinal toxicity, electroretinography is the gold standard for the detection of changes in retinal function. However, abnormalities are not detected in electroretinograms unless 20% or more of the retina is affected by the disease [151].

The retina is composed of multiple light-sensitive neuronal layers and contains the retinal pigment epithelium (RPE), photoreceptors cells (rod and cone cells), bipolar cells, horizontal cells, amacrine cells, ganglion cells, and Müller cells. Many miRNAs have been found in the retina and studied, and their retinal expression has been reviewed in several articles [29,152]. miR-124–3p, miR-96–5p, miR-182–5p (rat form: miR-182), miR-183–5p, and miR-204, and miR-211–5p are the most abundant retinal miRNAs. miR-96–5p, miR-182–5p, and miR-183–5p are members of the miR-183/96/182 cluster, and they are conserved across bilaterian

organisms [153]. In mice, the miR-183/96/182 cluster is enriched in the sensory organs including retina [154], inner ear [155], and olfactory bulb [156], but not in other organs/tissues [154,156]. In normal adult mice, miR-96–5p, miR-182–5p, and miR-183–5p are the most abundant miRNAs in the retina [157]. They are strongly expressed in the photoreceptors and are expressed to a lesser extent in the horizontal-bipolar-amacrine cells of the inner nuclear layer (Fig. 1D) [29,154,158]. In rats, these three miRNAs are almost exclusively expressed in the eye (Table 3) [56] and miR-182 and miR-183–3p are enriched in the photoreceptors of the retina although the distribution of miR-96–5p in retinal cells has not been investigated [159]. In addition to rodents, their enrichment in the retina as compared to the RPE or choroid has been confirmed in humans [160,161], without spatial expression analysis in the retina. In monkeys, the three miRNAs are listed as eye-specific miRNAs [84]. miR-124–3p is highly conserved in vertebrates and generally enriched in the nervous system including brain, eye, olfactory bulb, and spinal cord; however, they are not expressed in the other tissues of mice [156]. In the eye of adult mice, miR-124–3p is present in all retinal neurons, with strong expression in the photoreceptors of the outer segments and inner segment but not in the RPE (Fig. 1D) [162]. In rats, miR-124–3p is abundant in the eye at a level similar to the brain (Table 3) [56]. However, the expression of miR-124–3p in the human eye is lower than that in the brain (Table 2, DASHR2.0: <http://dashr2.lisanwanglab.org/index.php>). Although another report described that miR-124–3p is enriched in the human retina compared to the RPE, its expression accounts for only 5% of the most abundant miRNA in the retina, miR-182–5p [160]. In monkeys, miR-124a-3p has been identified to be eye-specific, among the 27 organs/tissues tested; however, this analysis did not include the brain [84]. miR-204–5p and miR-211–5p are located on different chromosomes. However, in most mammals, their sequences differ by only one nucleotide and they share an identical seed region. The two miRNAs are almost exclusively expressed in the eyes of mice [156], rats [56], and humans (DASHR2.0: <http://dashr2.lisanwanglab.org/index.php>). In the eye, they are distributed in many regions, particularly the RPE, lens, ciliary body, and horizontal-bipolar-amacrine cells in the inner nuclear layer, with weaker expression in the ganglion cell layer and photoreceptors [163,164] (Fig. 1D). In monkeys, miR-211–5p is listed as an eye-specific miRNA although detailed data are unavailable [84]. Lastly, the distribution of retinal miRNA has not been reported in dogs.

In rat retinal toxicity models, the levels of different miRNAs have been shown to be elevated in circulation, corresponding to the death of a specific retinal region (Table 4). In rats administered *N*-methyl-*N*-nitrosourea (MNU) at a dosage of 50 mg/kg, moderate photoreceptor cell death was observed on day 2, and an increase was seen in the serum miR-96–5p (43-fold), miR-183–5p (37-fold) and miR-124–3p (271-fold) levels as early as 6 h after administration, compared to time-matched vehicle-treated rats. In these rats, the amplitudes of the a- and b-waves in the electroretinograms decreased at 6 h, suggesting that the three miRNAs could be used as biomarkers for photoreceptor cell death [165]. Another research group reported on rat models of retinal toxicity induced by pan-cyclin-dependent kinase (CDK) inhibitors, AG-023422 and AG-012986. In these models, the rats were administered AG-023422 and AG-012986 intravitreally (30 µg/eye, single). On day 14, moderate retinal degeneration was observed as a result of AG-023422 administration, and moderate to marked degeneration of photoreceptors, outer plexiform layer, outer nuclear layer, and inner nuclear layer was observed as a result of AG-012986 administration. In these rats, post AG-023422 administration, the plasma miR-96–5p (~7–8-fold), miR-182 (~4-fold), miR-183–5p (~4-fold), and miR-124–3p (~50-fold) levels increased on day 3 and returned to

baseline on day 4, with the exception of miR-124–3p, which remained elevated on day 4. Similarly, in the AG-012986-treated rats, maximum elevation in the levels of plasma miRNAs (miR-96–5p: ~10-fold increase; miR-183–5p: ~6-fold increase; miR-124–3p: ~300-fold increase) was observed on day 3, with the exception of miR-182. The level of miR-124–3p remained elevated on day 7, while the levels of miR-96–5p and miR-183–5p returned to baseline [166]. In rats treated with *N*-methyl-D-aspartate (NMDA), slight to moderate retinal degeneration or necrosis of ganglion cells and inner nuclear layer cells were induced at 6 h, without RPE abnormality. In these rats, the serum miR-124–3p level increased 23-fold at 6 h, whereas the serum miR-96–5p, miR-183–3p, miR-204–5p, and miR-211–5p levels did not [165]. This is probably because miR-124–3p is enriched in NMDA-targeting cells including ganglion cells and inner nuclear layer cells (bipolar cells, horizontal cells, and amacrine cells). These reports from two independent research groups suggest that miR-124–3p is the most sensitive miRNA biomarker for retinal neuronal toxicity.

RPE is another component of the retina that supports photoreceptors by taking up glucose, retinol, and fatty acids from the blood, and supplying them to the photoreceptors; therefore, RPE dysfunction results in photoreceptor injury or death [167]. The oxidizing agent, sodium iodate (NaIO<sub>3</sub>), is thought to directly injure RPE by generating reactive oxygen species with secondary effects on photoreceptors [168]. In NaIO<sub>3</sub>-treated rats (30 mg/kg, i.v., single), slight to moderate RPE degeneration or death was observed on days 2 and 9; however, the levels of serum miR-204–5p and miR-211–5p did not change despite their abundance in these cells [165]. In these rats, electroretinograms exhibited a significant decrease at day 9, suggesting that these miRNAs may be less sensitive biomarkers compared to electroretinograms. Although the single i.v. administration (30 mg/kg) of NaIO<sub>3</sub> in rats did not induce any change in the serum miRNA levels [159,165], repeated doses (30 mg/kg/day for 2 days) increased the serum miR-96–5p (~5-fold), miR-182–3p (~4-fold), miR-183–3p (~2-fold), and miR-124–3p (~25-fold) levels on day 3, which returned to normal on day 5 [159]. This is because the photoreceptor cells, in addition to RPE, were severely damaged under the effect of repeated doses. Similarly, in cynomolgus monkeys administered i.v. NaIO<sub>3</sub>, the plasma miR-182–3p (up to 4.4-fold) and miR-183–3p (up to 6.9-fold) levels increased [169]. Taken together, circulating miRNAs are promising biomarkers for the diagnosis of retinal toxicity during nonclinical drug development, with miR-96–5p, miR-182–3p, and miR-183–5p being photoreceptor cell-specific biomarkers and miR-124–3p being a retinal neuron cell biomarker.

#### 5.4. Pancreas toxicity

Drugs account for less than 5% of all acute pancreatitis cases, with a variety of drugs being associated with pancreatitis including azathioprine, 6-mercaptopurine, didanosine, valproic acid, and mesalamine [170]. In addition, immune checkpoint inhibitor-induced pancreatitis and type 1 diabetes mellitus have recently been described [171,172]. Pancreas is composed of three major tissues: the exocrine acinar cells that produce digestive enzymes, and the centroacinar cells, ductules and ducts (ductal tree) that transport the digestive enzymes, and the hormone-containing endocrine islets consisting of 4 cell types producing insulin ( $\beta$  cells), glucagon ( $\alpha$  cells), somatostatin ( $\delta$  cells), and the pancreatic polypeptide (PP cells) (Fig. 1E). The amylase and lipase enzymes derived from the acinar cells are widely used as biomarkers for pancreas injury in humans and experimental animals. However, there are several drawbacks for the use of amylase and lipase as biomarkers for pancreatic injury. First, they have a short half-life in blood: 7–14 h for lipase and 9–18 h for amylase in humans [173],

and 1–2 h in rats [174]. The specificity of these enzymes is another issue. In addition to the pancreas, serum amylase can also be released from the salivary glands, small intestine, and liver [175]. On the other hand, the release of serum lipase occurs from the tongue, gastric mucosa, liver, muscles, and adipose tissues [176].

Certain pancreas-specific or -enriched miRNAs have been reported. Studies have shown that miR-217–5p, miR-216a-5p, and miR-216b-5p are exclusively expressed in the pancreas and localized in the acinar cells, but not in the islet cells, in both humans and rats [177,178]. Conversely, miR-375–3p is enriched in the islet cells of humans (Table 2) [179], rats [177], and mice [180], compared to the acinar cells (Fig. 1E) although miR-375–3p is also seen in the gastrointestinal tract (Tables 2 and 3). Pancreatic miRNAs in circulation have been used as biomarkers for drug-induced acute pancreatic injury in experimental animals (Table 4). These biomarkers include, miR-216a-5p [181], miR-216a-5p, miR-375–3p [54,182–184], miR-216a-5p, miR-216b-5p, miR-217–5p, and miR-375–3p [57] in rats, miR-375–3p [185], miR-216a-5p, miR-216b-5p, and miR-375–3p [177] in mice, and miR-216a-5p, miR-216b-5p, miR-217–5p, miR-375–3p [57], miR-216a-5p, and miR-375–3p [186] in dogs. During acute pancreatic acinar cell injury in rats, induced by 3 intraperitoneal doses of caerulein (50 µg/kg), administered 1 h apart, pancreatic necrosis was observed at 8, 24, and 48 h, while the levels of serum amylase (~2.5 fold) and lipase (~7-fold) increased during 1–8 h. In these rats, the levels of the 4 pancreatic miRNAs, miR-216a-5p (~7.5-fold), miR-216b-5p (~4-fold), miR-217–5p (~9-fold), and miR-375–3p (~4-fold) increased in the serum and remained elevated for longer periods of time (1–24 or 48 h) compared to the levels of amylase and lipase [57]. Another study of caerulein-treated (50 µg/kg, i.p., single) rats reported a much higher increase in the level of serum miR-217–5p at 4 (100-fold), 24 (1000-fold), and 48 h (1500-fold), while miR-216a-5p (90-fold), miR-216b-5p (100-fold), amylase (7.5-fold), and lipase (93-fold) increased only at 4 h [54]. Although the reason behind the large differences in the dynamic ranges of miRNAs is unknown, miR-217–5p appears to be superior to miR-216a/b-5p as a biomarker of pancreatic acinar cell injury in rats. This is because miR-216a/b-5p exhibit only a transient increase, depending on the study [54], probably due to the lower abundance of miR-216a/b-5p in the pancreas compared to miR-217–5p [178]. In another model of pancreatic toxicity, streptozotocin-induced islet β cell injury, the level of the islet-abundant miR-375–3p elevated in circulation at 4 h (40-fold) and 24 h (5-fold), and returned to baseline at 48 h, whereas the serum levels of the acinar cell-specific miR-217–5p, miR-216a-5p, and miR-216b-5p, as well as amylase and lipase produced in the acinar cells, remained unchanged [54]. It should be noted that the level of miR-375–3p in streptozotocin-treated rats returned to baseline at 48 h when an almost complete loss of islet cells was observed, suggesting the clearance of the released miR-375–3p from circulation and the lack of any viable cells that could release the miRNA. Serum insulin has been proposed as a biomarker for islet injury. However, its sensitivity is questionable, because the level of serum insulin decreased only after severe damage to the islet β cells [187,188]. Collectively, miR-375–3p can be detected in circulation during islet β cell injury, which makes it an early sensitive biomarker for the detection of type 1 diabetes mellitus [189].

In one of the most recent studies, the relative performance of the 4 pancreatic miRNAs (miR-216a-5p, miR-216b-5p, miR-217–5p, and miR-375–3p) as well as the activities of amylase and lipase in circulation were evaluated for the detection of acinar cell injury in rats induced by 5 pancreas toxicants and 10 non-pancreas toxicants with toxicity to other tissues [178]. They reported the superior performance of the 4 pancreatic miRNAs with ROAUC values of 0.98 (miR-217–5p), 0.90 (miR-375–3p), 0.82 (miR-216b-5p), 0.82 (miR-

216a-5p) compared to lipase (ROAUC: 0.79) and amylase (ROAUC: 0.74), for use as biomarkers of acute pancreatic acinar cell necrosis or degeneration. In particular, miR-217–5p showed the best performance among the all biomarkers tested. However, the levels of neither the 4 pancreatic miRNAs, nor amylase and lipase, elevated in rats with pancreatic fibrosis induced by a combination of caerulein/ethanol/cyclosporine A [178]. Therefore, the 4 pancreatic miRNAs in circulation add value to the interpretation of amylase and lipase activities for monitoring pancreatic injury in nonclinical studies. Collectively, miR-217–5p and miR-375–3p can be used as biomarkers for pancreatic acinar cell injury and islet β cell injury, respectively.

## 6. Conclusions

In this article, we have reviewed the emergence and progress of research on circulating miRNAs as biomarkers for drug-induced toxicities. Since their discovery in 2008, the potential utility of tissue-specific miRNAs as safety biomarkers of numerous tissue injuries has been demonstrated through basic research in both experimental animals and humans. The next stage will involve their use in the preclinical and clinical drug development processes as well as in clinical practice, to accelerate drug development and to sensitively monitor the toxicological events. Before being approved for use in drug developmental programs, every biomarker must pass a rigorous regulatory qualification process based on scientific data [190]. One of the major challenges is the uncertainty of the appropriate measurement and analytical procedures for the estimation of circulating miRNAs. The handling of serum/plasma, RNA extraction procedure, detection method, and normalization can affect the interpretation of the results. Another challenge is the large individual variation in the levels of the circulating miRNAs, as observed for miR-122–5p in humans [92]. The use of such a prospective biomarker in the clinical setting may be challenging. Therefore, besides biomarker evaluation studies, it is important to identify the potential factors (ex. age, race, sex, habit, diet) that cause variability in circulating miRNAs. Since a number of studies published so far have recognized circulating miRNAs with better sensitivity and specificity, we believe that overcoming these uncertainties will help circulating miRNAs to not only promote the preclinical drug development process, but also improve the monitoring of tissue injuries in clinical pharmacotherapy.

## Declaration of competing interest

The authors declare no conflicts of interest regarding the content of this article.

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