



Development and regulatory application of microRNA biomarkers

MicroRNAs, a class of regulatory small non-coding RNAs, are emerging as promising biomarkers for different health outcomes. Due to their tissue specificity, stability in extracellular space and high conservation between preclinical test species, applications of novel miRNA-based biomarkers for drug safety testing regarding hepatotoxicity and cardiotoxicity are investigated. Furthermore, miRNA expression is altered by environmental exposure such as cigarette smoke or polychlorinated biphenyls. As a consequence, miRNAs potentially influence tumor suppressor genes and oncogenes and may influence carcinogenesis. This has raised the interest in the use of miRNA profiles for health risk assessment. This review summarizes the recent developments in miRNA research with focus on biomarkers for drug safety testing and biomarkers for health outcomes related to environmental exposures.

Keywords: biomarker • cardiotoxicity • circulating miRNAs • drug safety testing • environmental exposure • hepatotoxicity • microRNA

The US FDA defines a genomic biomarker as “a measurable DNA and/or RNA characteristic that is an indicator of normal biologic processes, pathogenic processes and/or response to therapeutic or other interventions” [1]. Biomarkers currently available for determining toxic effects still have several shortcomings, including lack of sensitivity, specificity and straightforward biological significance. As a consequence, available biomarkers are not able to predict or eliminate all risks related to drug use in the early stages of drug development [2]. Furthermore, predicting disease risk from environmental exposures would benefit greatly from more sensitive and reliable biomarkers. While current toxicological biomarkers are often proteins, genomic elements may be used as indicators of toxicity earlier after the exposure, since it is an upstream event in the central dogma of molecular biology [3]. To this end, transcriptomics has become of great interest in biomarker research.

The human genome is almost completely transcribed, but only 1–2% of the transcripts

encodes for functional proteins [4,5]. The remaining transcripts are noncoding RNAs which have a wide range of regulatory functions. This class of RNAs can be subdivided into long noncoding RNAs, small interfering RNAs, microRNAs, Piwi-interacting RNAs, promoter-associated RNAs and enhancer RNAs. MicroRNAs (miRNAs) are small (≈ 22 nt), evolutionarily conserved, endogenously expressed single-stranded RNA molecules (for review see [5]). They are connected to the complex regulatory network of gene expression, exhibiting regulatory roles in a several biological processes [6]. Therefore, a connection between miRNAs and disease or toxicological exposures is obvious. This makes them interesting candidates for biomarkers in different toxicological applications.

This review will focus on the use of miRNAs as biomarkers in different regulatory applications, namely drug safety testing and health risk assessment as a consequence of environmental exposures. An increasing number of studies is being published, dem-

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onstrating the potential advantages of miRNA-based biomarkers over conventional biomarkers. In this review, we summarize these new findings and describe on how the use of miRNAs may improve regulations in both fields of application.

Biology of miRNA, a complex regulatory network

It is estimated that as much as 30–50% of mammalian genes exhibit miRNA-mediated regulation through complementary binding of the miRNA to the mRNA target [5]. One miRNA can regulate up to a hundred of different mRNA targets and one single mRNA target can be regulated by multiple miRNAs, adding a new dimension to the already complex network of gene regulation [7].

MiRNAs exert their function mostly by preventing protein synthesis through interfering with mRNA (Figure 1). However, elucidating the actual mechanisms by which miRNAs act is quite a challenge and there is no consensus about their mode of action (for review, see [6,8–9]). They can silence gene expression at the post-transcriptional level by translational repression, mRNA degradation and/or mRNA sequestration, but it is still unclear if and how these processes are linked [7–8,10]. Additionally, miRNAs do not only act as translational repressors. Through mRNA sequestration, miRNAs are also able to re-activate previously suppressed mRNAs [7]. Furthermore, they are able to activate gene expression through a link with the epigenetic machinery [11]. Finally, counter-regulation by circular RNAs can sequester miRNAs away from their target, thereby preventing their silencing effects [12].

Some of the contradictions in miRNA regulatory mechanisms may reflect real differences due to biological effects under certain conditions, but others might be artificial differences caused by alternative detection methods (e.g., microarray vs sequencing) [9].

miRNA quantification methods

RT-qPCR is the most common method for quantifying specific miRNAs. This technique is highly sensitive, well established and cheap as compared with the alternative methods. Different protocols have been reviewed (Table 1) [15]. If one seeks to measure a broad set of miRNAs simultaneously, RT-qPCR arrays combine the sensitivity of RT-qPCR with the possibility to quantify hundreds of miRNAs within a few hours.

The most established high-throughput approach is based on microarray technology, capable of quantifying the expression of thousands of nucleic acids simultaneously. There are currently different platforms

commercially available. Some major platforms have been reviewed and compared against each other using TaqMan RT-PCR as a reference [18]. Initiatives like the microarray quality control (MAQC), minimum information about a microarray experiment (MIAME) and external RNA control consortium (ERCC) provide standardization to microarray technology [24]. Furthermore, databases providing complete microarray datasets are publicly available, including miRNA microarray data, such as the Gene Expression Omnibus (GEO) [25] and ArrayExpress [26]. For large-scale population studies dealing with dozens of samples, microarray technology is the method of choice as one can simultaneously quantify thousands of miRNAs at relatively low cost. However, this technology holds several limitations, such as background noise signals, cross-hybridization, unequal melting temperatures or signal saturation, that are to be considered while designing an experiment [19].

High-throughput sequencing (HTS) represents nowadays a more comprehensive, sensitive and increasingly cost-effective approach, that overcomes many of these limitations. Furthermore, it allows screening of the complete miRNA spectrum, including low abundant and novel miRNAs as well as isomiRs and sequence modifications. Several HTS platforms are coexisting on the market and have been reviewed [21]. Small RNA sequencing protocols are available for the Illumina HiSeq, Roche 454 and SOLiD platforms [22]. Equivalent to the MAQC initiative, the Sequence Quality Control (SeQC) project seeks to establish standards for HTS [27]. Publicly available data from small RNA sequencing experiments can be retrieved from several databases such as the European Nucleotide Archive (ENR) [28] or the Sequence Read Archive (SRA) [29]. A complete workflow for miRNA quantification by Illumina Sequencing has been recently published [30]. While HTS is a robust technology, some technical issues should be considered when analyzing the data. For instance, enzymatic reactions during the library preparation could be prone to sequence and structure variations and consequently leading to biased miRNA quantification [31].

Several studies observing the same compound have reported non corresponding miRNA profiles, for example miR-21 has been described both up- and downregulated upon smoking [32]. This inconsistency can partly be explained by the use of different platforms. Results from sequencing as well as microarray experiments are prone to different biases leading to inconsistent miRNA quantifications. Differences within a platform can also influence the outcome of a study as for instance results from various available microarray platforms can vary depending on surface

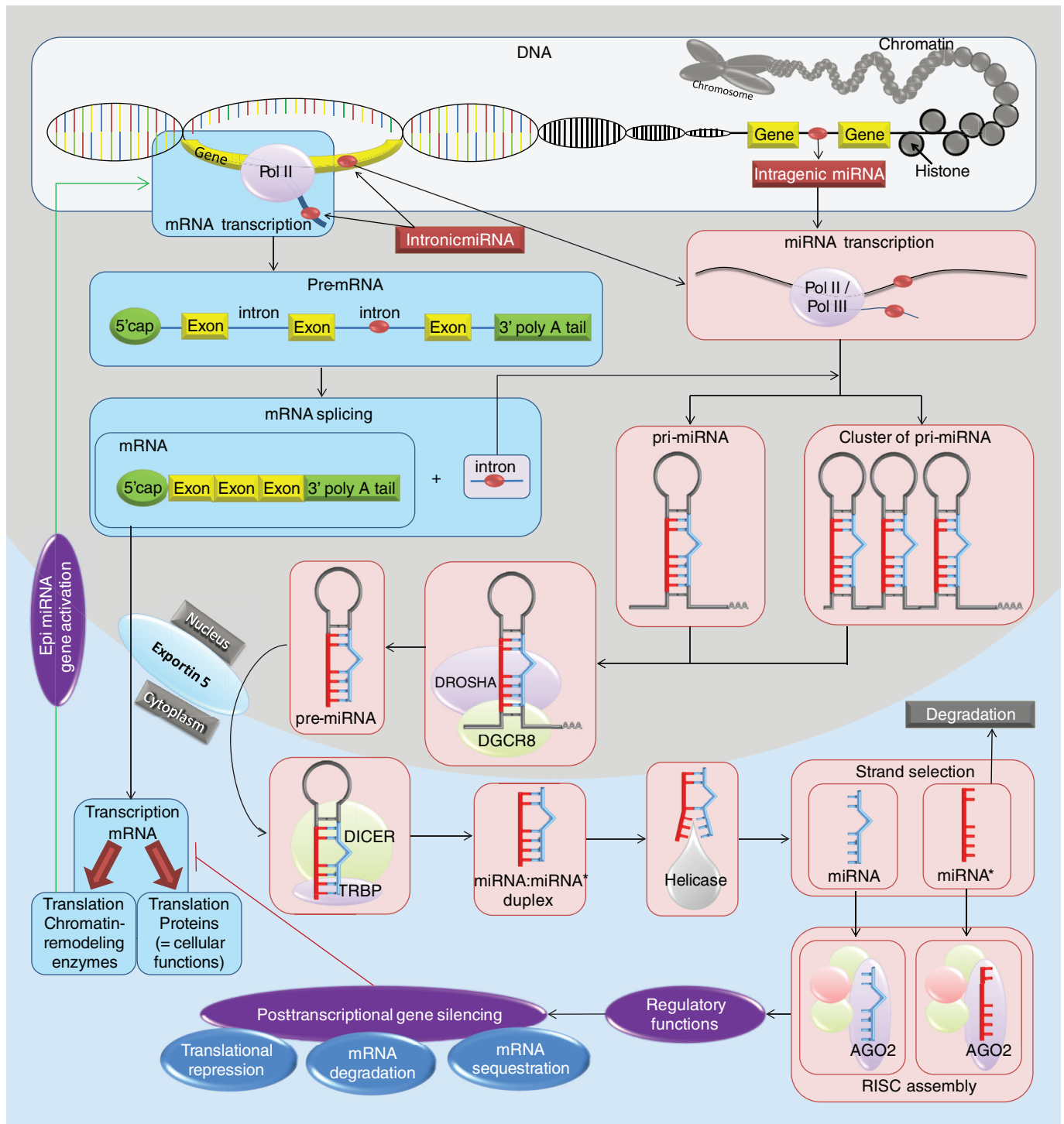


Figure 1. MiRNA biogenesis and regulatory function. Before a miRNA can exert its regulatory role within the cell, a primary miRNA (pri-miRNA) is initially transcribed from the genome by Pol II and processed by DROSHA into a precursor miRNA (pre-miRNA). After being transported to the cytoplasm, the pre-miRNA is cleaved by DICER into two mature miRNAs that can be incorporated into the RNA-induced silencing complex (RISC). Within this miRISC complex, mature miRNAs are responsible for target recognition via the AGO2, through target sites that are usually located in the 3'UTR of the targeted mRNA and involving often multiple binding sites. In mammals, miRNAs bind with mismatches and bulges, except for a key region of 7nt with perfect Watson–Crick base pairing called the 'seed' (for review, see [4,10,13–14]).

chemistry, printing technology, probe design or labeling techniques. Therefore, results should be verified by

other methods such as the reliable and cost-effective RT-qPCR [33].

Table 1. High-throughput methods of miRNA quantification.				
Technology	Platform	Ref.	Database	Application
RT-qPCR	Exiqon TaqMan Qiagen	[15]	-	Specific miRNAs; Validation [16,17]
Microarray	Affymetrix Agilent Illumina Exiqon	[18,19]	http://www.ncbi.nlm.nih.gov/geo/ https://www.ebi.ac.uk/arrayexpress/	Large-scale experiment [16,20]
High-throughput sequencing	Illumina HiSeq Life Technology/SOLiD	[21,22]	http://www.ncbi.nlm.nih.gov/sra/ http://www.ebi.ac.uk/ena	Novel and Low abundant miRNAs; Sequence modifications; complete miRNA spectrum [23]

Once a set of miRNAs has been identified as differentially expressed, the possible targets of these miRNAs can be investigated. For this purpose, a number of computational algorithms such as TargetScan (<http://www.targetscan.org/>) [34] or miRanda (<http://www.microRNA.org>) [35] are available. Furthermore, there are publicly available databases providing experimentally validated miRNA targets such as miRTarBase (<http://mirtarbase.mbc.nctu.edu.tw/>) [36].

MiRNAs as potentially good biomarkers

Since miRNA expression patterns change after exposure to toxic compounds, altering their target’s mRNA transcription and protein translation, they contribute to adverse biological effects and are thus promising candidates for toxicological biomarkers. Their expression patterns are organ-, tissue- or cell-specific, and are modified by compound exposure in a time and dose dependent manner [37–39]. Finally, an additional advantage is their high conservation in both humans and animal models which makes them widely applicable and allows for comparisons between animal experiments and clinical studies.

While the majority of miRNAs represents intercellular regulators involved in stress responses and environmental stimuli reaction, miRNAs are also detected in extracellular fluids (e.g., blood and urine [38,40]). MiRNAs in blood are referred to as circulating miRNAs, which are secreted into the circulation (either actively or passively) during the process of cytotoxicity which may ultimately result in cell death. Circulating miRNA are highly stable due to binding to other molecules such as AGO-proteins, or their incorporation in circulating macrovesicles or exosomes protect them from degradation [38,40–41]. Since vesicles generally have a functional role in cell-to-cell communication, circulating miRNAs may also play a role in signaling [41]. Their high stability combined with low expression level variation in healthy individuals makes them potential toxicological biomarkers.

Moreover, miRNAs possess several technical advantages as compared with other biological macromol-

ecules: they are structurally less complex, are reliably extracted and detected in serum or plasma, and they are resistant to repetitive freeze thaw cycles [40,41]. Finally, a clinically relevant aspect is their accessibility through noninvasive methods (e.g., through blood analysis), making them ideal for diagnosing disease, as well as for toxicological studies on chemical exposure effects and drug safety assessments in human population studies [38,42].

Applications of miRNAs as biomarker

We will now focus on two particular fields of miRNA applications, namely drug safety testing and environmental exposure. The first section will elaborate on recent developments of several miRNAs as biomarkers for drug-induced liver and heart injuries. The second section will focus on recent achievements in unraveling the complex involvement of miRNAs in health outcomes as a consequence of environmental exposures.

Drug safety testing

Drug development and safety assessment are long and costly processes, which are currently not able to eliminate all risks related to drug use. Drug safety testing is therefore focused on finding the balance between the predicted risk and the expected clinical benefit for the patient [2,43].

The drug discovery process can be divided in several parts (Figure 2) [43,44]. Many investigated candidate compounds fail before entering clinical trials because of lack of clinical efficacy or adverse drug reactions (ADRs) [45]. Since not all toxicities are detected, ADRs are a major cause for regulatory actions such as drug withdrawal from the market and restrictions on drug use in the form of black box warnings [40,43,46]. Furthermore, drug induced toxicities rank high as cause of disease and death. In these cases, hepatotoxicity and/or cardiotoxicity were the most frequently reported, indicating that there is still need for improvement in the field of drug safety testing [47]. It would be ideal if a candidate drug could be reliably tested for the possibility of drug induced toxicity early in the discovery process.

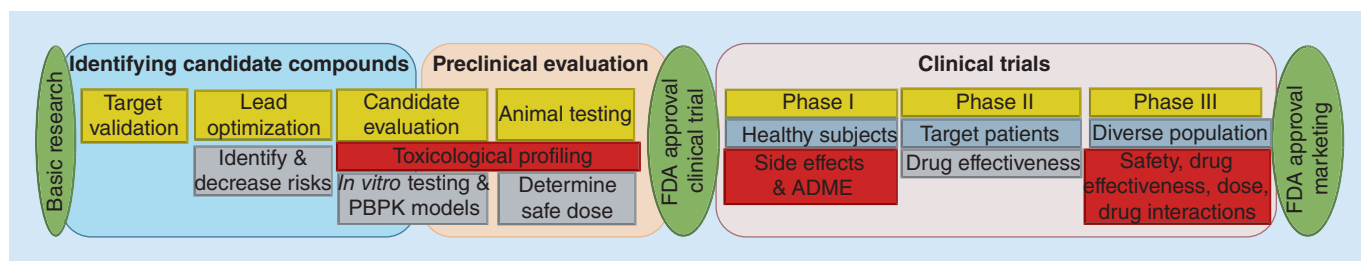


Figure 2. Overview of the drug development process. Parts within the process that would greatly benefit from improved toxicity biomarkers are indicated in red.

ADME: Absorption, distribution, metabolism, and excretion of the tested drug; PBPK: Physiologically based pharmacokinetic model.

Being able to reduce the two primary causes of toxicity (hepatic and cardiac) would already have a major impact on reducing risk during clinical trials and thus identifying miRNA biomarkers able to detect toxicity in these two organs are a major goal in the field [2].

MiRNA in drug-induced liver injury (hepatotoxicity)

Drug-induced liver injury (DILI) or hepatotoxicity is associated with severe outcomes and represents the leading cause of acute liver failure in the USA [46,48]. It is defined as “a liver injury caused by various medications, herbs, or other xenobiotics, leading to abnormalities in liver tests or liver dysfunction with the reasonable exclusion of other etiologies” [49]. Since DILI comprises a broad classification, it results in diverse clinical manifestations [46] that may resemble almost any known liver disease be it through different underlying mechanisms [50]. The majority of DILI cases are classified as idiosyncratic and can occur up to three months after drug administration. This unpredictability not only hampers the diagnosis of DILI, but also causes problems during drug development. Therefore, sensitive and specific biomarkers for liver injury are necessary [40,51–52]. Furthermore, individual susceptibility also plays an important role in DILI. Predisposing factors comprise ethnicity, concomitant liver diseases (especially non-alcoholic fatty liver disease), nutritional status and diet (e.g., Type 2 diabetes mellitus, obesity and the metabolic syndrome), age and gender [51,53]. However, the main cause of DILI is the use of acetaminophen (also known as APAP or paracetamol). APAP is a safe drug when taken at therapeutic dose, but since it is an over-the-counter analgesic and antipyretic drug it too often leads to overdosing with potentially fatal hepatocellular necrosis and acute liver failure [52]. Because APAP-induced acute liver failure occurs frequently, DILI research is mainly focused on this compound.

Relevance of new biomarkers in DILI

Current preclinical testing strategies are not able to reliably predict DILI. It is estimated that 20–40%

of potential DILI-causing drugs remain undetected in preclinical pharmaceutical evaluation [41,46]. It is therefore important that research focuses on the development of predictive tests using data from diverse sources in order to identify DILI causing drugs during development and, ultimately, prevent DILI risk in patients [39,46,52].

Conventional biomarkers of liver injury include ALT, AST and bilirubin [40,52]. Of these markers, bilirubin is an indicator of altered liver function, while ALT and AST are ‘leakage’ biomarkers that indicate disruption in hepatic cell and tissue integrity [41]. However, these biomarkers are neither sensitive nor specific [42,48]. The markers provide a useful indication for liver damage when substantial, and sometimes irreversible, tissue damage has occurred [41]. Furthermore, serum transaminases also increase in other extra-hepatic injuries [40]. Thus, new biomarkers for DILI diagnosis in the clinic and preclinical settings would be of great value.

Examples of DILI miRNA biomarkers

Previous research has shown the possibility of evaluating DILI by measuring circulating miRNA expression (Table 2), and identified miR-122, miR-192, miR-193 and miR-155 as relevant candidates [48].

Currently the most promising miRNA biomarker candidate for DILI is the liver specific miR-122 [3]. MiR-122 is a highly conserved miRNA that comprises approximately 70% of all miRNAs in a healthy liver [23,41]. Notably, it regulates gene networks involved in lipid metabolism and cell differentiation [41]. During liver injury, hepatocytes may release miR-122 in blood circulation [48]. However, miR-122 may also influence liver regeneration after sustaining injury from, for example, toxic exposures [52]. In mice with APAP induced DILI, the upregulation of this miRNA was dose- and exposure duration dependent. Compared with the conventional ALT biomarker, miR-122 shows greater sensitivity in the sense that it could be detected earlier (6 h after exposure instead of 24 h) and at lower APAP doses [3,54]. MiR-122 has also a greater tissue-specificity compared with ALT.

Table 2. Upregulated miRNAs in liver and heart toxicity.					
miRNA	Indicator of	Toxicant	Sample	Species	Ref.
miR-122	Liver injury	APAP	Serum	Rat	[54]
			Plasma	Mouse	[3]
					[55]
		Ethanol Trichloro-bromomethane		Human	[56]
					[23]
				Mouse	[55]
miR-192	Liver injury	APAP	Serum	Rat	[54]
			Plasma	Mouse	[3]
				Human	[56]
	Diabetic nephropathy				[58]
miR-193	Liver injury	APAP	Plasma	Mouse	[3]
			Serum	Rat	[54]
miR-194	Liver injury	APAP	Serum	Human	[23]
miR-210					
miR-483					
miR-375	Type 2 diabetes	None	Serum	Mouse	[59]
	Tissue injury (incl. liver)	APAP	Serum	Human	[60]
miR-940	Liver injury				[60]
Urine panel (375, 940, 9 and 302)			Urine		
Urine panel (20b, 34c, 185, 291a, 296, 330, 433, 434, 484, 664)		Liver injury	APAP	Urine	Rat [61]
miR-155	inflammation	APAP and ethanol	Plasma	Mouse	[55]
miR-146a		APAP			
miR-125b					
miR-208	Myocardial injury	Isoproterenol	Plasma	Rat	[62]
		Isoproterenol and doxorubicin			[63]
miR-208a/b	AMI	None		Human and rat	[64]
miR-208b	AMI and VM			Human	[65]
miR-499	AMI	None	Plasma	Human	[66]
				Human and rat	[64]
				Human and mice	[67]
	AMI and VM			Human	[65]
miR-1	AMI	None	Plasma	Human	[68]
				Human and rat	[64]

AMI: Acute myocardial infarction; VM: Viral myocarditis.

miRNA	Indicator of	Toxicant	Sample	Species	Ref.
miR-1 (cont.)			Urine	Rat	[46]
miR-133a	AMI	None	Plasma	Human and mice	[67]
				Human and rat	[64]
				Human	[69]
miR-206	Skeletal muscle injury	Isoproterenol and doxorubicin	Plasma	Rat	[63]

AMI: Acute myocardial infarction; VM: Viral myocarditis.

Where ALT levels increased also in the case of extra-hepatic injury, concomitant miR-122 upregulation was not observed [70]. Although most analyses of miR-122 have been conducted in rodents, the utility of miR-122 as biomarker has been confirmed in human studies [23,56,71]. MiR-192, and the less investigated miR-193, are other liver-enriched miRNA which resemble miR-122 in their reaction to liver injury, although with a smaller dynamic range and these may also be good candidate biomarkers [3,54].

The aforementioned miRNAs alone cannot discriminate between benign liver injuries and more serious, life-threatening conditions [48]. Because APAP, and possibly other drugs, induce necrosis often accompanied by inflammation, it might also be beneficial to include miRNA biomarkers related to inflammation during drug safety testing, thereby creating a miRNA panel to assess liver damage. Examples of inflammation-related miRNAs that have been found to be time-dependently upregulated in the circulation during DILI are miR-155, miR146a and miR-125b [55]. The miRNA panel can be further expanded by including more recently found circulating miRNAs that are upregulated during DILI: miR-194, miR-210, miR-483 [23], miR-375 and miR-940 [60].

However, miR-122 and miR-192 are generic biomarkers for liver injury, independently of the cause [40], since they have also been reported to be upregulated in non-drug-induced liver diseases [70,72]. A possible solution would be to use blood plasma fractionation in order to distinguish between different types of liver injury, as circulating miRNAs, including miR-122 and miR-155, were found in the exosome fraction of alcoholic liver disease and in the protein-rich fraction during DILI [73]. Finally, since preclinical testing is not limited to non-invasive methods for miRNA detection, it is noteworthy that most miRNAs found over-expressed in the circulation, show decreased expression levels in tissue due to leakage or active transport of these miRNAs [3].

MiRNA in drug-induced heart failure (cardiotoxicity)

Drug-induced heart failure or cardiotoxicity is also a major concern for drug safety testing. In human, cardiotoxicity is mostly caused by anticancer drugs, and prescription drugs of other classes (e.g., antibiotics, antidepressants, antipsychotics) [74].

Relevance of new biomarkers in drug-induced heart failure

Drug-induced cardiotoxicity poses many similarities regarding the need for new biomarkers as DILI. First, it manifests in many different ways, ranging from subclinical myocardial injury (development of asymptomatic cardiac abnormalities) to clinical heart failure symptoms [75]. Second, drug-induced cardiotoxicity is an important cause for drug withdrawal from the market [40], accounting for approximately 45% of total post-approval prescription drug withdrawals [74]. And third, current biomarkers are neither specific nor sensitive enough for timely diagnosis, making prevention and/or reduction of cardiac damage difficult. Thus, patients treated with cardiotoxic drugs may suffer from undetected long-term side effects, which may lead to additional morbidity and even mortality [40,75].

Conventional serum biomarkers of myocardial injury include cardiac myoglobin, creatine kinase-MB isoenzymes, natriuretic peptides and cardiac troponins: cTnT, cTnI and cTnC [2,40,74]. Among these biomarkers, cardiac troponins are currently considered as the 'gold standard' for diagnosis of acute myocardial infarction (AMI) [64]. During myocardial injury, these cTns are released from cardiomyocytes upon injury or death, thereby increasing the serum levels [2,74]. A major problem of these biomarkers is that they are not heart-specific and can also be found in other muscle types. Therefore, it is often difficult to distinguish between cardiac injury and exercise [2,75]. Also, low sensitivity, and therefore failure to detect toxicity in

early stages, is a clear indication that development of novel biomarkers is still required [63,75].

Examples of drug-induced heart failure miRNA biomarkers

As for DILI, myocardial injury can be assessed by evaluating circulating miRNA expression (Table 2). Subsequently, miR-208, miR-499, miR-1, miR-133 and miR-206 have become important targets in this research field [40,63].

MiR-208 is encoded by an intronic region of the *Myh6* gene and regulates the myosin heavy chain isoform switch. In pathological conditions, it is involved in cardiac remodeling [76]. In isoproterenol-exposed rats, plasma levels of miR-208 and cTnI were highly correlated and exhibited similar time courses. Because cTnI may also be upregulated during renal dysfunction, miR-208 and cTnI were also compared in bilaterally nephrectomized rats. This did not demonstrate miR-208 upregulation, indicating that miR-208 plasma levels might be a superior biomarker to cTnI for detection of myocardial injury in rats [62]. In humans, the plasma levels of several miRNAs were analyzed, including the miR-208 family, comprising the closely related miR-208a and miR-208b. MiR-208a was detectable in AMI patients, while being undetectable in healthy subjects and non-AMI patients (including acute kidney injury, chronic renal failure, stroke and trauma). Furthermore, it can be detected earlier than cTnI (1–4 h after myocardial injury instead of 4–8 h). However, as compared with AMI animal models, miR-208a and miR-208b concentrations in the human heart appeared to be much lower [64,66–67]. Therefore, an optimized RNA isolation protocol for low abundance miRNAs is necessary [64]. Overall this suggests that miR-208 is highly specific for cardiac tissue injury and allows the timely detection of myocardial injury.

Several studies showed that miR-499, miR-1 and miR-133a were upregulated in AMI patients (Table 2). MiR-499 promotes differentiation of cardiac progenitor cells into myocytes and may play a role in the activation of repair mechanisms following AMI. MiR-1 and miR-133 play opposing roles in cell fate determination; miR-1 being pro-apoptotic, while miR-133 is anti-apoptotic. Both are also involved in regulation of cardiac electrical properties [67]. One drawback of these three miRNAs, especially of miR-1 and miR-133a, is that their expression is even higher in skeletal muscles than in heart [64]. To overcome this drawback, miR-206, a skeletal muscle-specific miRNA, might be used to correct for muscle injury in order to assess the myocardial injury [63]. All of these miRNAs were reported to be highly sensitive early biomarkers for detection of cardiac injury [66,68]. Furthermore, plasma levels

of miR-499 changed more profoundly than miR-1 and miR-133a, which may reflect higher specificity of miR-499 for cardiac muscle [65].

Environmental exposure

It is widely accepted that environmental exposures present an important human health threat. The World Health Organization (WHO) estimated 7 million deaths due to air pollution exposure in 2012, not including the fatal outcomes from tobacco smoke. These findings confirm that air pollution is the world largest environmental health risk [77]. During the last decade, several studies have demonstrated the impact of environmental exposure on gene expression profiles [78], DNA-methylation patterns [79] and p53 status [80]. More recently, the interest in the effect of environmental exposures on miRNA expression is rising; the question has emerged whether or not miRNAs may serve as a reliable biomarker for environmental exposure. To this end, some recent publications assessed the expression of miRNA in different environmental exposure conditions such as cigarette smoke [81], diesel exhaust particles (DEPs) [82] or polychlorinated biphenyls (PCBs) [16].

The first evidence that environmental exposures may also influence the expression of miRNAs was provided by a study in which rats were exposed to cigarette smoke and which identified 24 significantly downregulated miRNAs in lung tissue. These included the lung cancer-associated miRNAs let-7, miR-34 and miR-125 [83]. In a follow-up study, the effect of cigarette smoke was studied in mice with a comparable outcome of 15 downregulated miRNAs which also comprised let 7 and miR-125 [84]. In humans, Schembri *et al.* compared miRNA expression in the bronchial epithelium between ten never smokers and ten current smokers and observed 28 significantly differentially expressed miRNAs [81]. The majority of these 28 miRNAs, again including let-7, confirmed a pattern of downregulation of miRNAs by cigarette smoke (see Table 3).

The most commonly inhaled environmental pollution is particular matter (PM), an exposure which is related to various adverse health outcomes, including the reduction of pulmonary function, lung cancer and cardiovascular diseases [95]. Several *in vitro* and *in vivo* studies have been conducted to explore the effect of PM on miRNA expression and to investigate whether the effect is comparable to the effect of cigarette smoke. Diesel exhaust particles (DEPs), consisting of a complex mixture of hundreds of agents, are the main source of emitted PM and exposure is strongly associated with inflammation and histopathological changes in the lung as well as increased lung cancer risk [96]. An *in vitro* study investigated the miRNA expression in human epithelial cells and observed 130 upregulated

Table 3. Exposure related miRNAs and association with human cancer.

miRNA family	Exposure association	Species	Sample	Ref.	Cancer association
Let-7	Cigarette smoke	Rat	Lung	[83]	Lung cancer [85]
		Mouse	Lung	[84]	
	Asbestos	Human	Lung	[20]	
	PCBs	Mouse	P19 cells	[16]	
miR-21	PM	Human	Blood	[17]	Lung cancer [85]
	iAs	Human	Umbilical vein endothelial cells	[86]	Lymphoma [87]
	PCBs	Mouse	P19 cells	[16]	Breast cancer [88]
miR-34	Cigarette smoke	Rat	Lung	[83]	Lung cancer [85]
	NNK	Rat	Lung	[89]	Lymphoma [87]
miR-125	Cigarette smoke	Rat	Lung	[83]	Breast cancer [88]
		Mouse	Lung	[84]	Ovarian cancer [90]
		Human	Lung	[81]	
miR-150	iAs	Human	Jurkat cells	[91]	Lymphoma [87]
miR-155	PCBs	Mouse	P19 cells	[16]	AML [92]; lymphoma [87]; breast cancer [88]
miR-181	BaP	Human	HepG2 cells	[93]	Lymphoma [87]
	iAs	Human	Jurkat cells	[91]	
	PCBs	Mouse	P19 cells	[16]	
miR-199	Asbestos	Human	Lung	[20]	Ovarian cancer [90]
	NNK	Rat	Lung	[89]	Lung cancer [94]
miR-222	Cigarette smoke	Rat	Lung	[83]	Lung cancer [85]
	PM	Human	Blood	[17]	
	iAs	Human	Jurkat cells	[91]	

and 67 downregulated miRNAs [97]. Several miRNAs were similarly affected upon DEPs and cigarette smoke exposure in human. However, in contrast to the effect of cigarette smoke, the majority of the miRNAs was upregulated indicating an exposure-specific miRNA response [98]. Steel plant workers, exposed to metal-rich PM, observed significantly increased blood levels of the lung cancer-associated miRNAs miR-21 and miR-222 after one working week [17].

Asbestos-related lung cancer is one of the leading occupational cancers. Thirty-four miRNAs were identified to be significantly altered in an experiment exploring miRNAs in tumor and corresponding normal lung tissue from highly asbestos-exposed and nonexposed patients. This miRNA profile, including the lung cancer-associated miRNA let-7 and miR-199, classified the normal samples into one group and the tumor samples into two separate groups respective to the exposure. Furthermore, eight novel asbestos-related miRNAs were identified that distinguished all asbestos-exposed from nonexposed patients' samples.

Next to that, the study explored transcriptomic data in these samples and found inversely correlated gene targets that are known to play a role in carcinogenesis [20].

Several epidemiological studies associate exposure to benzo(a)pyrene (BaP) with an increased risk of numerous forms of cancer, including tumors in liver, lungs and oral cavity [99]. Humans are exposed to BaP by polluted air, but also by the intake of contaminated food and water. A study analyzed the time-dependent effects of BaP on mRNA and microRNA profiles in a human liver cell line and revealed eight miRNAs that appear to participate in specific BaP-responsive pathways relevant to genotoxicity, such as apoptotic signaling, cell cycle arrest, DNA damage response and DNA damage repair [93].

Inorganic arsenic (iAs) is a natural occurring metalloid that exists at high levels in drinking water in endemic regions. This contamination is of major concern as it affects several hundreds of people worldwide. It has been identified to induce lung, skin and bladder cancer as well as vascular injury [100]. An *in vitro*

study exposing human umbilical vein endothelial cells observed alternations of miRNA expression that possibly play a crucial role in vascular injury [86]. A profile consisting of 36 miRNAs involved in cell cycle progression and apoptosis, was significantly altered by iAs exposure, supporting the idea of iAs carcinogenic activity [91].

Polychlorinated biphenyls (PCBs) are synthetic organic compounds that, in the past, were widely used in industry and electronic devices. Due to concerns about health and environmental risks, PCBs were banned in the USA in 1977 and classified as a human carcinogen in 2003 [101]. PCBs are highly persistent, have been distributed widely in our environment and may accumulate after intake or exposure in fatty tissues of humans and animals. Therefore, human population is still exposed to PCBs by air and food. Besides several studies that show the effect of PCBs on transcription level [102], Zhu *et al.* provided the first evidence that exposure to PCBs also changes the miRNA expression profile by comparing PCB-exposed mouse embryonal carcinoma cells and control cells in a microarray experiment and observed 45 differentially expressed miRNAs [16]. Among these, the expression of the cancer-associated miRNAs let-7, miR-21, miR-155 and miR181 was significantly altered. In a recent *in vivo* study, miR-191 expression in human peripheral blood mononuclear cells was significantly correlated with blood concentrations of dioxin-like PCBs [103].

Exposure to genotoxic compounds may lead to DNA damage, but this does not necessarily result in cancer as damaged DNA can be repaired by enzymatic repair systems or eliminated by means of apoptosis. If these protective mechanisms do not function adequately and the DNA-damage leads to mutations in the genome, a cell may become cancerous, especially if the mutation takes place in a tumor-suppressor gene or oncogene. MiRNAs regulate cancer-related processes such as cell growth, DNA repair or apoptosis by targeting relevant tumor-suppressor genes or oncogenes. As shown above, carcinogenic compounds can alter the expression of miRNAs and may lead to upregulation of oncomiRs or downregulation of oncoprotective tumor suppressors and hence to cancer.

After 1 week of treatment, rats that were exposed to tobacco-carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) showed a downregulation of several miRNAs, including miR-199 and miR-34 [89]. This miRNA profile shows a considerable overlap with altered miRNAs in human lung cancer samples [98]. Long-term exposure to cigarette smoke induced irreversible alterations of miRNA expression in mice and led to an irreversible downregulation of let-7, targeting the lung cancer related oncogene *KRAS*.

Consequently, this event led to a rise in expression of mutated *KRAS* and induced carcinogenesis in the lungs [84]. Also miR-125, a miRNA targeting *ERBB2* proto-oncogene that encodes the EGF receptor, was downregulated in this study [104]. Both studies observed a significant downregulation of miR-34 which is a key regulator of tumor suppression. It controls the expression of numerous genes involved in cell cycle, differentiation and apoptosis, and antagonizes processes that are necessary for basic cancer cell viability as well as cancer stemness, metastasis and chemoresistance [105].

Another promising biomarker candidate is miR-155, which is highly associated with lymphoma [87], acute myeloid leukemia (AML) [92] and breast cancer [88]. In a study examining PCB-exposed mouse embryonal carcinoma cells, a significant upregulation of miR-155 was observed [16]. A key target of miR-155 is the transcription factor PU.1, which is involved in leukemogenesis. Knock-out mice with suppressed PU.1 expression die as a result of an aggressive leukemia-like disease at an age between 3 and 8 months [106]. Besides the potential for a prognostic biomarker, miR-155 also emerges as a new target for cancer therapy [92].

Altered expression of miR-21 has been observed as a result of PM and iAs exposure. Recently, it has been found that miR-21 regulates *BTG2* during carcinogenesis. A tumor suppressor gene that is involved in many biological activities such cell differentiation, proliferation, DNA damage repair and apoptosis in cancer cells [107].

Future perspectives on miRNA biomarkers

In order to be used as a clinical biomarker, miRNA detection should be robust, quick, simple, accurate, reproducible and inexpensive, which is not the case as of yet. Furthermore, there are still gaps in our understanding of miRNA-mediated gene regulation. Since miRNAs can reduce the amount of mRNA through three different mechanisms and may simultaneously also activate gene expression, the precise impact of miRNA on disease risk often remains unclear. Furthermore, it is unknown whether circulating miRNAs leak from damaged tissues or if they also get actively secreted as a form of cell-to-cell communication. Thus, for application in a clinical setting or in the context of drug safety testing, it would be beneficial to clarify these uncertainties in miRNA mode of action. Still, miRNA biomarkers may already provide useful information to increase insight into molecular mechanisms of toxicity, when used as additional toxicity tests. MiR-122 and miR-208 are specially promising new biomarkers for detection of DILI and myocardial injury, respectively. Since these miRNAs are general indicators of liver or heart injury, their

plasma level increase does not indicate the cause of the toxicity. Therefore, a panel of miRNA to assess ADRs may, among others, also include inflammation-related miR-155 and skeletal muscle-specific miR-206. The most ideal way to further assess the applications of these (and other) miRNAs as drug toxicity biomarkers would be a comprehensive investigation on toxicant-induced modulations of miRNAs in tissues treated by a large number of toxicity-inducing drugs. To this end, in the EU/FP7 Hepatic and Cardiac Toxicity Systems (HeCaToS) project, liver and heart 3D microtissues will be exposed to a large number of hepatotoxic and cardiotoxic compounds, respectively, to elucidate toxicological mechanisms by combining cross-omics analysis with functional assays and bioinformatics tools [108]. Data generated by this project will contribute to the unraveling of underlying mechanisms of toxicity, and to the identification of biomarkers for the assessment of hepatotoxicity and cardiotoxicity. This research can benefit utility assessment of miRNA biomarkers and may identify novel ones to expand the proposed miRNA biomarker toxicity panel for drug safety testing.

Exposure-specific miRNA patterns have also been revealed, such as a general downregulation of miRNAs upon cigarette smoking [81], and a general upregulating upon PM exposure [97]. Furthermore, it has been found that not only the dose but also the duration of exposure are reflected in the miRNA expression [104]. The reviewed miRNA profiles may eventually lead to improved health risk assessments. However, these studies did not find the same miRNAs per compound and some miRNAs have been described as both up- and downregulated by the same compounds. Part of the inconsistency might be explained by different cell-lines, organisms or exposure models but also by varying quantification methods. Therefore, further experimental studies are needed to identify compound-specific effects on the miRNA machinery and to clarify the role of miRNAs within the complex interplay of exposure and carcinogenesis. Cross-omics studies, integrating different platforms such as miRNA, transcriptomic and methylation data, show great potential in assessing the impact of environmental pollutants to health outcomes and will bring advantages over single-omics biomarkers. Next to that, further population studies

Executive summary

Relevance of novel miRNA biomarkers

- Conventional biomarkers for determining toxic effects still have several short comings, including lack of sensitivity, specificity and straight forward biological significance.
- MiRNAs are emerging as promising biomarkers because:
- Expression patterns are organ, tissue or cell specific and modified by compound exposure in a time and dose dependent manner

Technical aspects of miRNA analysis

- Currently, there are three different high-throughput methods for miRNA quantification commercially available.
- RT-qPCR arrays, microarray technology and high-throughput sequencing, each having advantages for particular applications.

MiRNAs as biomarker for drug safety testing

- Drug safety testing is currently not able to eliminate all risks related to drug use.
- Adverse drug reactions are a major cause for regulatory actions such as drug withdrawal.
- Reducing the two primary causes of toxicity (hepatic and cardiac) would already have a major impact on lowering risks.
- Especially miRNA-122 and miRNA-208 are promising new biomarkers for detection of drug-induced liver injury and myocardial injury, respectively.

MiRNAs as biomarker for environmental exposure

- Environmental exposures present an important human health threat.
- MiRNAs are altered upon exposure to different environmental pollutants:
- These alterations can impact on the expression of tumor-suppressor genes or oncogenes and hence may promote cancer.
- Exposure-specific miRNA profiles have been revealed including cancer related miRNAs such as miR-34 and miR-155.

Future perspective

- Before miRNA biomarkers can replace conventional diagnostic tools in a clinical setting, or during drug safety testing, gaps in our understanding of miRNA regulation have to be addressed.
- MiRNA biomarkers may already be of value to increase insight into molecular mechanisms of toxicity, when used as additional tests.
- The reviewed miRNA profiles may eventually lead to improved health risk assessments.
- More studies are needed to identify and validate novel miRNA-based biomarkers.

are required to identify and validate novel miRNA biomarkers and to clarify their advantages over conventional biomarkers for environmental exposure monitoring and health risk assessment.

Future perspective

With the development of both high-throughput sequencing technologies, becoming cheaper and faster every day, and the emergence of personalized medicine, in the near future we may very well witness a decrease of the need of single end point biomarkers. Indeed, by generating the full miRNA expression profile of a patient sample, it may become possible to compare

the patients complete expression panel to a database of disease miRNA profile signatures and establish a diagnosis.

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