Dynamics and regulation of the oxidative stress response upon chemical exposure

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MicroRNA patterns as biomarkers for chemical exposure and disease

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ABSTRACT

MicroRNAs are small non-coding RNAs. They regulate gene expression at the post-transcriptional level and are involved in many biological processes. Recent studies indicate that circulating microRNAs are stable and can be assessed in a non-invasive manner, making microRNAs interesting biomarker candidates. Furthermore, microRNA expression changes are linked to various diseases like liver disease and cancer. In this review, changes in microRNA expression after chemical exposure are described. A literature study was conducted to obtain information related to microRNA up/down regulation after exposure to a diverse panel of chemicals. Interesting, the microRNAs most frequently found to be dysregulated are also found to play a role in various diseases linked to chemical exposure. Although microRNA expression changes show great potential as biomarkers, questions concerning biomarker robustness, biological functionality and adverse outcome causality of the response still remain.

INTRODUCTION

MicroRNA (or miRNA) is one of many types of non-coding RNA. MicroRNAs are small, around 22 nucleotides in length, and single stranded in their active mature form. Biogenesis of microRNAs contains multiple different steps. First, primary microRNA transcripts (pri-miRNA) are transcribed in the nucleus by RNA polymerase II or III. Next, each pri-microRNA is cleaved into hairpin loop structures (precursor microRNA or pre-miRNA) by the microprocessor complex DGCR8. The pre-miRNA is transported to the cytoplasm by Exportin-5 and cleaved into an imperfectly double-stranded microRNA by the RNAse III protein Dicer. The passenger strand is degraded and the guide strand is incorporated into a RNA-Induced Silencing Complex (RISC). MicroRNAs regulate gene expression at the post-transcriptional level through sequence-specific binding to target mRNAs (Pogribny et al. 2016). MicroRNAs are involved in many physiological processes including the immune response, metabolism, and development (Hou et al. 2011). Furthermore, microRNAs are involved in toxicological responses and disease. Moreover, microRNAs are highly conserved among species (Lewis et al. 2003). Because of their imperfect complementary binding to mRNA, a single microRNA is able to target many different mRNAs (Bolleyn et al. 2015; Mohr and Mott 2015). Although the function of microRNAs is mostly described as downregulation of their target genes, microRNA binding will frequently not result in complete silencing of genes. In fact, sometimes only minor changes in protein response can be observed (Cech and Steitz 2014). Therefore microRNAs might be better described as "fine-tuners" of biological processes. The fact that the working mechanism of microRNAs is not a simple matter of on-off, complicates matters concerning their role in health and disease. Furthermore, a single microRNA might have opposing functions in different systems (Mohr and Mott 2015). Another aspect worth mentioning here is that different microRNAs can "work together" in downregulating one single mRNA target (Mohr and Mott 2015). For example, miR-375, miR-124, and let-7b were found to work together in enhancing myotrophin targeting in the pancreas, resulting in greater repression of the corresponding mRNA target (Krek et al. 2005).

Because of their role in a broad range of physiological processes, microRNAs can provide us with numerous information. Concerning chemical exposure, microRNAs might provide an indication of the working mechanism (mode-of-action) of the chemical. Since some microRNAs demonstrate highly "tissue specific" expression, measurement of these microRNAs as surrogate biomarkers in medium in *in vitro* test systems or plasma in experimental animal models and humans might provide information regarding the target organs that are damaged upon chemical exposure and ensuring release in extracellular fluids (Laterza et al. 2009). Also, different microRNAs are described in literature which are related to (various types of) cancer

Table 1. MicroRNA patterns after chemical exposure.

MicroRNAs specific for the target organ are depicted in green. MicroRNAs known to be organ specific, but not for the organ/tissue where they have been measured are depicted in red. ↑= upregulated, ↓ = downregulated.

Chemical compound	Species	Tissue/cell type/ body fluid	Altered miRNA	References
Ethanol	Rhesus macaques	PBMC	181a↑, 221↑	(Asquith et al. 2014)
		Colon	155↑	(Asquith et al. 2014)
	Rat	Gastric tissue	145↑, 17↓, 19a↓, 21↓, 181a↓, 200c↓	(Luo et al. 2013)
	Mouse	Liver	132↑, 155↑	(Bala and Szabo 2012)
	Rat	Liver	34a↑, 103↑, 107↑, 122↑, 19b↓	(Dippold et al. 2013)
	Mouse	Liver	34a↑	(Meng et al. 2012)
	Human	Hepatobiliary cell lines	34a↑	(Meng et al. 2012)
	Human	HEK239T cells	7↑, 144↑, 203↑,15b↓	(van Steenwyk et al. 2013)
	Mouse	Cerebellum	132↑, 155↑	(Lippai et al. 2013)
	Human	Monocytes	27a↑	(Saha et al. 2015)
	Human	SH-SY5Y neuroblastomal cells	302b↑, 497↑	(Yadav et al. 2011)
	Mouse	AML-12 hepatocytes and liver	217↑	(Yin et al. 2012)
	Human	Caco-2, intestinal epithelial cell	212↑	(Tang et al. 2008)
	C57BL/6 mouse	Liver	705↑,1224↑, 182↓, 183↓, 199a-3p↓	(Dolganiuc et al. 2009)
Aflatoxin	Rat	Liver	34a↑, 92↓	(Yang et al. 2014)
	Human	Chang liver	33a-5p↑	(Fang et al. 2013)
	Human	HepG2 liver carcinoma cells	33a-5p↑	(Fang et al. 2013)
Arsenic	Human	Jurkat leukemic T cell	30d↑, 142-5p↑, 150↑, 181a↑, 222↑,638↑, 663↑	(Sturchio et al. 2014)
	Human	HXO-RB44 Retinoblastoma cells	34a↑, 125a-3p, 129-5p↑, 181b↑,425↑, 628-3p↑, 649↑,890↑, 943↑, let-7b↓, 220b↓ 376a↓, 524-5p↓	(Zhang et al. 2013)
	Human	HUVEC	19b↑, 21↑, 24↑, 29b↑, 33a↑, 301a↑, 874↑, 198↓, 508-5p↓, 1252↓	(Li et al. 2012)
	Human	T24 bladder carcinoma cells	222↑, 19a↓	(Cao et al. 2011)
	Human	HepG2 liver carcinoma cells	24↑, 29a↑, 30a↑, 210↑, 886-3p↑, 296-5p↓, 663↓, 675↓, 744↓	(Meng et al. 2011)

Chemical compound	Species	Tissue/cell type/ body fluid	Altered miRNA	References
	Rat	Liver	151↑, 183↑, 26a↓, 423↓	(Ren et al. 2015)
	Human	Bronchial epithelial cell	21↑	(Luo et al. 2015)
	Human	A549 lung cell	98↑	(Gao et al. 2014)
	Human	NB4 acute promyelocytic	125↑, 126↑, 193b↑, 215↑, 335↑	(Ghaffari et al. 2012)
		leukemia cell	372↓	(Ghaffari et al. 2012)
	Human	Hepatocellular carcinoma cells	491↑	(Jiang et al. 2014)
	Human	Hepatocellular carcinoma cells	491↑	(Wang et al. 2014)
	Guinea pig	Myocardium	1↑, 133↑	(Shan et al. 2013)
	Human	Bronchial epithelial cells	190↑	(Beezhold et al. 2011)
Acetaminophen	Porcine	Plasma	122↑,124-1↑, 192↑	(Baker et al. 2015)
	Human	Serum	9-3p↑, 30d-5p↑, 122↑, 125b-5p↑, 204-5p↑, 423-5p↑,574-3p↑, 4732-5p↑	(Yang et al. 2015)
	Human	Urine	302a↑, 357↑, 940↑, Let-7d*↑, 188-5p↑, 197↑	(Yang et al. 2015)
	Mouse	Liver	207↑, 297a↑, 297b-3p,328↑, 466c-5p↑, 466d-3p↑, 466f-3p↑, 466g↑, 466g↑, 467a*↑, 467b*↑,467e*↑,468↑, 574-5p↑, 574-3p↑, 483↑, 483*↑, 485*↑, 669a↑, 669c↑, 671-5p↑, 672↑, 689↑, 709↑, 710↑, 711↑, 721↑, 877*↑, 1224↑, Let-7b↓, 29b↓,29c↓, 30a↓, 101b↓ ,212↓, 122↓, 129↓, 130a↓, 192↓, 194↓, 487b↓	(Wang et al. 2009)
	Mouse	Plasma	Let-7d*↑, Let-7g↑, 15a↑, 19b↑,21↑, 22↑, 27b↑, 29a↑, 29b↑, 29c↑, 30a↑, 30c↑, 30e↑,101b↑, 107↑, 122↑, 129↑, 130a↑, 148a↑, 192↑, 294*↑, 365↑, 574-5p↑, 680↑, 685↑, 23a↓, 26a↓, 124↓, 125a-3p↓, 125b-5p↓, 133a↓, 133b↓, 135a*↓,202-3p↓, 205↓, 451↓, 468↓,	(Wang et al. 2009)

Chemical compound	Species	Tissue/cell type/ body fluid	Altered miRNA	References
			483↓,710↓, 711↓, 712↓, 720↓, 721↓, 1224↓	
	Human	Blood	19a↑, 19b↑, 374a↑	(Jetten et al. 2012)
	Rat	Liver	298↓, 370↓	(Fukushima et al. 2007)
Benzo(a)pyrene	Human	HepG2 liver carcinoma cells	26a-1↑, 29b↑, 140-5p↑, 181a↑, 542-5p↑,	(Lizarraga et al. 2012)
			1271↑, 1973↑, 122↓, 448↓, 518e↓, 518e↓,	
			582-3p↓, 591↓, 2276↓	
	Human	HepG2 liver carcinoma cells	181a-3p↑	(Caiment et al. 2015)
	Rat	Liver	29b↑, 34b-5p↑,34c↑, 21↓, 122↓, 142-5p↓,	(Chanyshev et al. 2014)
			221↓, 222↓, 429↓	
	Rat	Ovary	21↑	(Chanyshev et al. 2014)
	Mouse	Liver	34a↑	(Malik et al. 2012)
	Mouse	Liver	140↑, 207↑, 290↑, 291a-3p↑, 346↑, 376b↑,	(Zuo et al. 2014)
			483↑, 292-3p↓, 433-5p↓, 489↑, 434-3p↓, 546↓	
	Mouse	Colon	290↑, 291b-5p↑, 292-5p↑, 298↑, 351↑,	(Zuo et al. 2014)
			433-5p↑, 503↑, 546↑	
	Mouse	Glandular stomach	207↑, 290↑, 291b-5p↑, 292-5p↑, 298↑, 346↑,	(Zuo et al. 2014)
			351↑, 433-5p↑, 503↑	
	Mouse	Lung	207↑, 290↑, 291a-3p↑, 291a-5p↑, 376b↑,	(Zuo et al. 2014)
			434-3p↑, 489↑, 291b-5p↓, 292-5p↓, 433-5p↓	
	Mouse	Spleen	207↑, 290↑, 291b-5p↑, 292-5p↑, 298↑, 376b↑,	(Zuo et al. 2014)
			346↑, 351↑, 542-5p↑, 464↓	
	Mouse	Forestomach	291b-5p↑, 292-5p↑, 298↑, 346↑, 351↑, 503↑,	(Zuo et al. 2014)
			546↑, 140↓, 199b↓, 207↓, 433-5p↓, 489↓	
	Mouse	Lung	150↓	(Halappanavar et al. 2011)

(Meng et al. 2016) and other pathologies like acute myocardial infarction (Devaux et al. 2012). Altogether, these features make microRNAs interesting candidates for biomarkers for chemical exposure and related pathological outcomes.

With respect to the use of microRNAs as biomarkers of chemical exposure and adverse outcomes, we questioned whether exposure to a chemical would display a compound specific (microRNA) response, providing information about the chemical mode-of-action, or that the microRNA would merely reflect a general target organ toxicity response. We reasoned that in case of a general toxicity response, some microRNAs linked to general toxicity outcomes such as cell apoptosis, will be frequently found to be differently expressed across different studies using different chemicals and *in vitro*/*in vivo* systems. Another question we wanted to address was whether the microRNAs that are differently expressed after chemical exposure are more specific to the test system than to the type of chemical exposure.

To answer these questions, we performed a systematic literature study to obtain information related to changes in microRNA expression after exposure to chemical compounds. We focused on several compounds for which sufficient microRNA expression information in diverse tissues and/or test systems is available in the literature and these included: ethanol, aflatoxin B1, arsenic, paracetamol (acetaminophen), and benzo(a)pyrene. We did exclude literature describing exposure to mixtures or teratogenic effects. Sources used are PubMed and Science Direct. Keywords for literature searches simply involved microRNA and the individual compound name. We further only focused on microRNAs that were additionally validated by qRT-PCR experimentation.

MICRORNA CHANGES AFTER CHEMICAL EXPOSURE, LEAKAGE FROM DAMAGED CELLS OR FUNCTIONAL RESPONSE?

From our literature search we obtained in total 39 manuscripts that fulfilled our criteria. We did not take any consideration on dose and time point along; this will be discussed later. In Table 1 all the results on microRNA expression after treatment to the five selected chemicals are summarized from these manuscripts. Data were obtained from *in vitro* human test systems and *in vivo* experiments in rodents. We also observed a diversity of target tissues, with arsenic being tested in various cell types *in vitro*, and benzo(a)pyrene being assessed in different tissues *in vivo*.

We further evaluated this combined dataset and as a first step we investigated the commonality in microRNA expression changes by the five chemicals. Table 2 summarizes the microRNAs that were differentially expressed upon exposure to at least two different study compounds. We observed at least seven microRNAs, miR-21, miR-34, miR-19, miR-26, miR-29, miR-122 and miR-181, that were affected by at least three of the five chemicals. Remarkably, miR-21 and miR-34 were differentially expressed after exposure to four of the five chemicals included in this study, suggesting that these microRNAs are part of general cellular stress response pathways that are modulated after cell injury upon chemical exposure. Interestingly, these microRNAs are extensively studied in relation to cancer: miR-21 is found to regulate processes like cell proliferation and apoptosis where overexpression of miR-21 increases cell viability (Tong et al. 2015), suggesting a role in a protective adaptive response upon chemical exposure. For some microRNAs that were in overlap between multiple compounds, we observed opposite effects by the different compounds. For example, ethanol showed increased expression of miR-122 in rat liver, yet was down-regulated in rat liver after acetaminophen; the latter coincided with increase of miR-122 in plasma. These contrasting effects might be due to severe liver cell damage, which results in leakage or active transport of miR-122 out of the cells. Given that miR-122 is a highly expressed liver specific microRNA (Wang et al. 2021), miR-122 is likely not directly involved in the damage response itself.

Expression changes are known to depend on chemical class and cell type or tissue (Rodrigues et al. 2011). To answer the question whether or not the microRNA changes found in this meta-analysis are tissue specific, the TSmiR (Tissue-Specific microRNA) database was used (http://bioeng.swjtu.edu.cn/TSmiR) (Guo et al. 2014). This database only contains information of human microRNAs, but considering the fact that Shan et al. (2013) found upregulation of miR-1 and miR-133, two microRNAs known to be tissue specific for heart tissue, in Guinea pig myocard tissue, might indicate that at least some of the tissue specific microRNAs are consistent across different species (Shan et al. 2013). MicroRNAs known to be specific for the following tissues were incorporated: bone, brain, heart, kidney, liver, lung, pancreas, skeletal muscle, spleen, and thymus. We observed a few tissue specific microRNAs that were affected by the compounds, including miR-1 and miR-133 for myocard, and miR-483 and miR-192 for liver (see Table 1, marked in green). The list of microRNAs known to be tissue specific, but observed in other tissues (see Table 1, marked in red), was more numerous and included micoRNAs that were observed either in cell lines *in vitro*, possibly because of higher sensitivity due to a single cell type as compared to tissue with mixed cell types. In addition, we observed such tissue specific microRNAs in serum, e.g. miR-9-3p, miR-124, miR-125b-5p and miR-192 or urine including miR-302a. These observations were particular made for acetaminophen which induces

hepatotoxicity. The liver injury biomarker miR-122 was not part of the list; this is likely due to low levels of miR-122 that are released from the liver into the blood under normal physiological conditions. If microRNAs leaked from severe damaged tissue are collected in the blood, they will be present in a higher amount than normal. Therefore, these microRNAs are likely good candidates for a biomarker of exposure. For example, a chemical substance known to be hepatotoxic, will damage the liver and in turn the blood will be enriched for liver specific microRNAs, such as miR-122 (Wang et al. 2021). Interestingly, Wang et al. (2009), found some microRNAs to be upregulated in mouse plasma, but down regulated in mouse liver after acetaminophen exposure (Wang et al. 2009). Furthermore, they also found microRNAs which were down regulated in plasma but upregulated in liver. The authors stated that their findings could be the result of cellular damage, but do not rule out the possibility of a specific transport mechanism. We speculate that most of the microRNAs that are released in the blood and urine, and derived from damaged tissues are not directly functionally involved in the damage response. Yet, there are indications that such microRNAs present in exosomes may indirectly impact the biology of the same tissue or cells in other tissues (Rahman et al. 2020). Altogether, these liver specific microRNAs that are released in the blood after acetaminophen might be used as biomarkers of exposure to hepatotoxic substances (Llewellyn et al. 2021; Wang et al. 2021).

Table 2. Differently expressed microRNAs by at least two different study compounds.

In this table subgroups of microRNAs are taken together. For example: miR-19a and miR-19b are shown as miR-19. Other subtypes are: 34a (all compounds except acetaminophen). 34b-5p benzo(a)pyrene. 34c benzo(a)pyrene. All miR-34 subtypes where found to be upregulated. miR-26: 26a/b, 26a alone and 26a-1. For miR-21 no subtypes where described in the used literature. miR-21 was down regulated in ethanol (rat gastric tissue) 1x up and 1x down regulated in acetaminophen and benzo(a)pyrene (mouse/rat tissue). Concerning arsenic exposure, three studies using different types of lung cells, all found miR-21 to be upregulated. MicroRNA shown are the microRNAs differently expressed by the chemical compound.

MicroRNA	EtOH	AFB1	As	APAP	BaP	Total nr. of chemicals
miR-21	✔		✔	✔	✔	4
miR-34	✔	✔	✔		✔	4
miR-19	✔		✔	✔		3
miR-26			✔	✔	✔	3
miR-29			✔	✔	✔	3
miR-122	✔			✔	✔	3
miR-181	✔		✔		✔	3
Let-7			✔	✔		2
miR-27	✔			✔		2
miR-30			✔	✔		2
miR-33		✔	✔			2
miR-92	✔	✔				2
miR-107	✔			✔		2
miR-125			✔	✔		2
miR-133			✔	✔		2
miR-142			✔		✔	2
miR-150			✔		✔	2
miR-183	✔		✔			2
miR-193			✔	✔		2
miR-199	✔				✔	2
miR-207				✔	✔	2
miR-212	✔			✔		2
miR-221	✔				✔	2
miR-222			✔		✔	2
miR-298				✔	✔	2
miR-302	✔			✔		2
miR-376			✔		✔	2
miR-423			✔	✔		2
miR-483				✔	✔	2
miR-1224	✔			✔		2

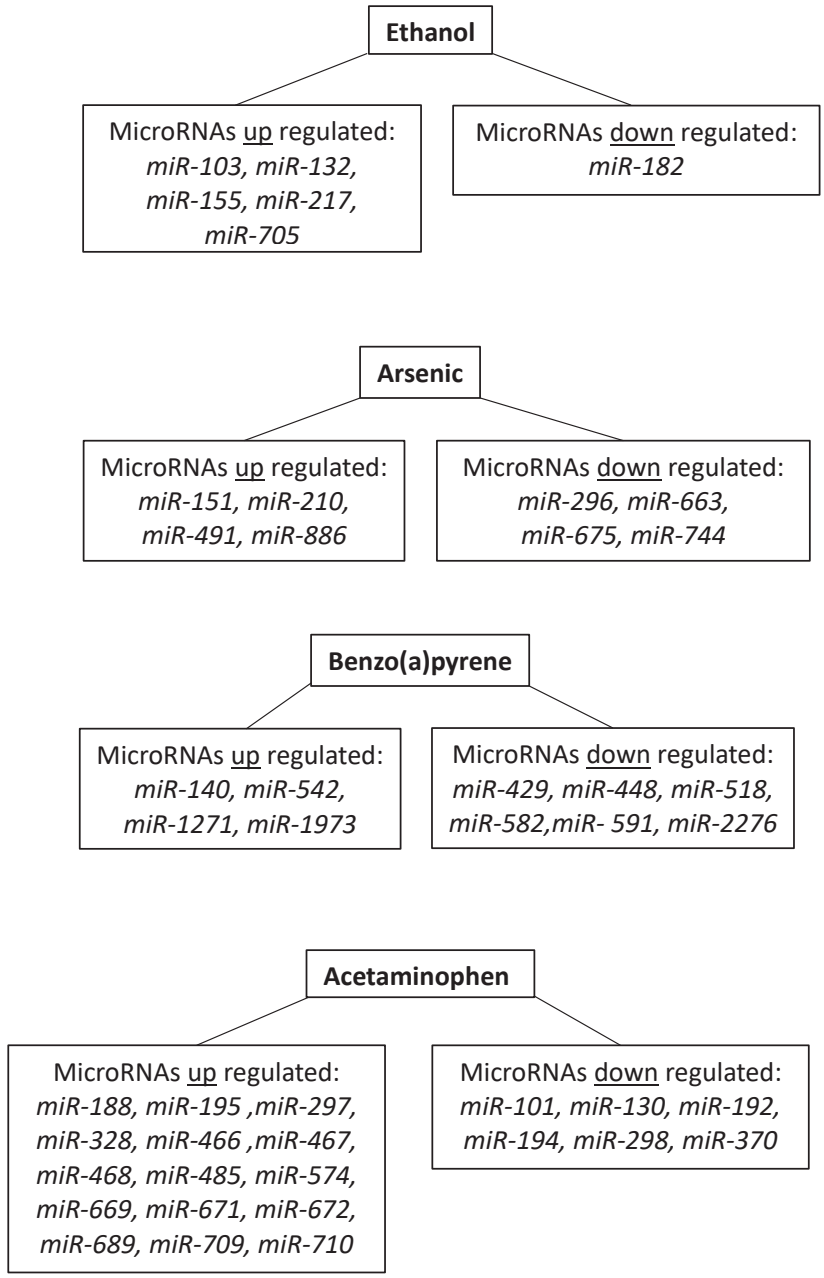

Figure 1. Up/down regulated microRNAs in liver tissue (human or animal) and *in vitro* liver models.

MicroRNAs depicted were only found to be dysregulated by only one of the five compounds. For aflatoxin, no unique microRNAs were found in liver tissue/cells.

UNIQUE EXPRESSED MICRORNAS AS BIOMARKERS OF EXPOSURE

Given the limited overlap in microRNAs that are in common between the five toxicants, we further focused on the compound specific microRNAs to derive an improved indication about the (specific) mode-of-action of the compounds. Since microRNAs will affect gene expression and thereby the overall biology, we selected up- and down-regulation for microRNAs which were only found to be differentially expressed by exposure to one single compound (Figure 1). Since liver was most studied, only results derived from *in vivo* liver tissue (human, rat or mouse) or *in vitro* liver models were combined. In these liver samples we observed highly treatment specific differentially expressed microRNAs patterns for the five compounds. To determine the feasibility to uncover biological connections of these compound specific microRNAs we performed a target prediction analysis. For this we focused on benzo(a)pyrene since its reactive metabolites will lead to covalent modification of DNA and consequently activate a DNA damage response. The target genes of the microRNAs found to be differentially expressed due to exposure to benzo(a)pyrene were determined in mirDB (http://www.mirdb.org/miRDB/). Only target genes with a target score of 100 were included. Next, the function of these target genes was derived using the NCBI gene database (https://www.ncbi.nlm.nih.gov/). While we identified four upregulated and six downregulated microRNAs, for only in total four we could derive predictions for target genes. For the upregulated microRNAs, miR-140 and miR-1271, only one and three target genes were predicted, and for miR-140 no biological information on this target gene was uncovered (see Figure 2). Interestingly, three genes, *ZEB1*, *ZEB2* and *TRIM33* reported to play a role in the DNA damage response, were found to be targeted by miR-249, one of the six downregulated miRNAs after Benzo(a)pyrene exposure (Kulkarni et al. 2013; Sayan et al. 2009).

This example of benzo(a)pyrene demonstrates the complex relationship between exposure, microRNA pattern changes, and changes at gene level, to provide mechanistic insight on the adverse effects. Also the lack of data, and the fact that there is no 100 % target score available for all microRNAs, makes it difficult to interpret the data from a mechanistic perspective. We are fully aware that interpreting and comparing microRNA data from different papers is still difficult because of the use of different biological test systems, different exposure times, compound concentrations and of course different microRNA analysis platforms and data normalization approaches. This also indicates the requirement that information obtained from different databases has to be combined. Given these constraints, there is a considerable uncertainty that the microRNAs we defined based on our

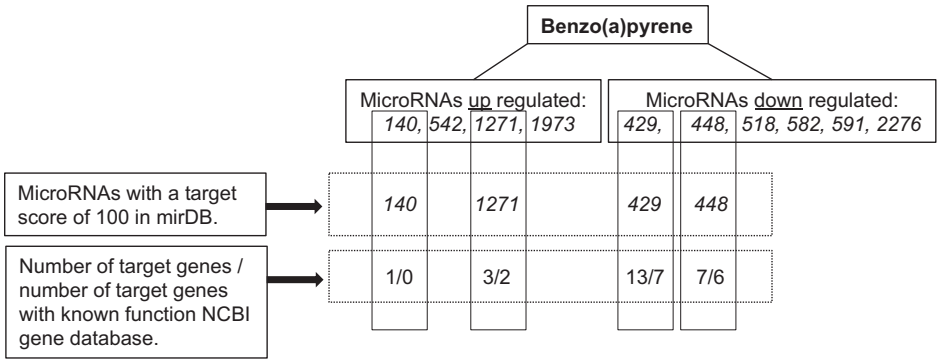

Figure 2. Overview of microRNAs differently expressed after benzo(a)pyrene exposure linked to information found in target prediction databases and NCBI.

MicroRNAs target genes with a target score of 100 found in mirDB. Their function is derived from the NCBI gene database. Not for all the target genes with a score of 100 a function was available in the NCBI database.

systematic meta-analysis may not be specific for the mode-of-action of the chemical compound. In addition, the identified single miRNA could have no relationship with the direct biological transcriptional and biological reprogramming after the chemical exposure in the target cells of toxicity. Firstly, the chemical of interest might indirectly impact the production and maturation process of microRNAs. This could be particularly true under conditions of severe cell injury that would disrupt normal cellular homeostasis and transcriptional processing. Secondly, microRNAs are thought to function by fine-tuning numerous processes (Mendell and Olson 2012). This implicates that the effect of a single microRNA will probably result in only a moderate effect at protein level. The effect of microRNAs will therefore rather be the resultant of a complex mechanism involving a set of different microRNAs. Lastly, microRNAs might have an indirect effect on their target genes (Lee et al. 2013). Once released from injured cells in the blood circulation, microRNAs may impact on the systemic biology through other means. Specific microRNAs, i.e. miR-21 and miR-29, can then bind to the toll-like receptor, triggering pro-inflammatory responses (Fabbri et al. 2012). Also, in plasma, microRNAs can be found encapsulated in vesicles and as Argonaute2 complexes (Arroyo et al. 2011). Argonaute2 proteins are the key effector proteins in microRNA-mediated silencing (Bartel 2018; Golden et al. 2017). Furthermore, microRNAs can also be delivered to recipient cells by high-density lipoproteins (Vickers et al. 2011). This alternative indirect biological effects of microRNAs make it difficult to interpret the specific functionality and, or causality of the microRNA response in relation to mechanisms of toxicity.

MICRORNA EXPRESSION PATTERNS AS BIOMARKERS OF DISEASE

Above we summarized the effect of five often studied chemicals on microRNA expression. Next, we questioned whether some of the key microRNAs found in our study are also known to be differentially expressed in diseases/adverse outcomes linked to the chemicals selected in this study. In the next session we focus on liver disease (ethanol, acetaminophen) and cancer (arsenic, benzo(a)pyrene, aflatoxin B1).

Liver disease

One of the most differently expressed microRNAs described after acetaminophen and also ethanol exposure is miR-122 (Figure 1 and 2) with a decrease in liver and coinciding increase in plasma and serum. miR-122 is the most abundant microRNA in human liver (Jopling 2012) and has therefore been proposed as a novel liver injury specific biomarker. miR-122 has specific biological activities. Thus, miR-122 plays a role in various processes like lipid, cholesterol metabolism and cell differentiation. Furthermore, miR-122 is found to suppress cell proliferation (Hu et al. 2012) and to promote hepatitis C virus replication (Hsu et al. 2012). Hepatocellular carcinoma cells have reduced levels of miR-122. Interestingly, HepG2 cells, a human hepatocellular carcinoma cell-line, known to be miR-122 deficient, was found to be able to take up exosomal miR-122 released by Huh7 cells when cultured together, reducing cell growth and proliferation in HepG2 cells (Basu and Bhattacharyya 2014); high levels of miR-122 in the liver hepatocytes may therefore maintain a non-proliferative differentiated phenotype. miR-122 also plays a role in inflammation: it downregulates cytokines like IL-6 and IL-1β in human hepatic stellate cells (HSCs) which is associated with an inhibition of the NF-κB nuclear translocation in human HSCs (Nakamura et al. 2015). Interestingly, miR-122 knock out results in the upregulation of the immunomodulating transcription factor RelB in the liver (Hsu et al. 2020). While miR-122 has been suggested as a single translational biomarker for liver injury; we also observed other microRNAs that show a decrease in liver and an increase in plasma after acetaminophen treatment, including miR-192 (Krauskopf et al. 2015). Furthermore, the use of a biomarker panel for drug induced liver injury consisting of miR-122 complemented with miR-192 and miR-193 is described (Su et al. 2012). Moreover, a broader "microRNA-based composite biomarker" for nonalcoholic steatohepatitis was suggested, containing ALT and miR-192, miR-21, and miR-505 (Liu et al. 2018), although we did not find these microRNAs together as a panel in any of the chemical treatments in our analysis. A recent study on the validation of various candidate liver injury biomarkers indicates the use of a combination of miR-122 with cytokeratin 18 (K18) and glutamate dehydrogenase (GLDH) in human subjects with liver injury (Llewellyn et al. 2021).

Cancer

In cancer, microRNAs might be separated into two groups: tumor-suppressive microRNAs and oncogenic microRNAs (Gao and Liu 2011). The various studies demonstrate in particular modulation of miR-34 and miR-21.

In our analysis, miR-34 was affected by four chemicals. miR-34 is a direct p53 target (Tian et al. 2014) and involved in stabilization of the p53 response to genotoxic stress and as such functions as a tumor-suppressive microRNA (Bommer et al. 2007; Navarro and Lieberman 2015). Three homologous of miR-34 are known: miR-34a, miR-34b, and miR-34c. In particular upregulation of different miR-34 homologues was observed in livers of rat and mouse after treatment with DNA damaging agents aflatoxin B1 and benzo(a)pyrene but not acetaminophen (see Table 1).

miR-21 was affected by ethanol, acetaminophen, arsenic and benzo(a)pyrene. miR-21 is one of the most studied oncogenic microRNAs. miR-21 is found to be upregulated in most cancer types (Kumarswamy et al. 2011). The enhanced expression of miR-21 in cancer cells has suggested this microRNA as a biomarker for malignancy in various tissues. miR-21 has a direct impact on the cytoprotection of cells to stress. Chan et al. (2005), found that knockdown of miR-21 in glioblastoma cells leads to the activation of different caspases and eventually to increased apoptosis and cell death (Chan et al. 2005). Although miR-21 clearly plays a role in many types of cancer, another function of miR-21 is related to signal transduction in T-lymphocyte, where miR-21 is a negative modulator of T-cell activation and its expression is induced in memory cells compared to naïve T-cells (Carissimi et al. 2014). Furthermore, miR-21 is found to be induced by inflammatory stimuli, like for example TGF-β1 (Haakensen et al. 2016; Wang et al. 2018). The p53 target gene B cell translocation gene 2 (BTG2) is a target for miR-21, and a possible mechanism by which cardiomyocytes are protected against doxorubicin, a cytostatic compound used for chemotherapy in the treatment of cancer (Tong et al. 2015). Moreover, Thum et al. (2008), investigated the role of miR-21 in cardiac fibroblasts and found that inhibiting miR-21 results in an increase in apoptosis. Overexpression of miR-21 showed the opposite result. As an explanation they propose a mechanism whereby miR-21 negatively regulates SPRY1, which leads to an increase of ERK activation, which in turn leads to cell survival (Thum et al. 2008).

Consistent with the above observations is that miR-21 and miR-34 can be used to differentiate between genotoxic and non-genotoxic carcinogenicity in mouse hepatocytes (Marrone et al. 2016; Rieswijk et al. 2015). Yet, caution should be taken since there is no strong consistency in direction and magnitude of microRNA expression changes after chemical exposure. Furthermore, these changes might also reflect general toxicity rather than a mechanism eventually leading towards

cancer. Moreover, Lu et al. (2005), hypothesize that, because in healthy tissues the overall expression of microRNAs seems to be higher compared to tumors, the global microRNA expression reflects the state of cellular differentiation (Lu et al. 2005).

DISCUSSION AND FUTURE CHALLENGES

We conducted a literature study in order to obtain learnings concerning the overlap and specificity of differential expression of microRNAs upon chemical exposure. We have observed a diversity of microRNAs for our selected five chemicals that have been investigated most. For some microRNAs overlap between expression was observed with miR-21 and miR-34 as important examples with modulation by four out of five compounds, which can partly be linked to either a DNA-damage stress response by e.g. benzo(a)pyrene, aflatoxin B1 and arsenic or a more general cellular stress response by ethanol and acetaminophen. The current studies prohibit to link any of the microRNA expression patterns to a specific type of chemical exposure. While integration of microRNA and mRNA for mechanistic interpretation has been applied for improved mechanistic understanding (Caiment et al. 2015; Rieswijk et al. 2015), experimentally validating the mechanistic hypothesis on such causative relationships has so far been lacking.

The use of microRNAs as biomarkers of exposure or disease looks promising. First of all because of the fact that microRNAs can easily be obtained in different body fluids like blood (serum/plasma), urine and saliva and are highly stable. Secondly, because microRNAs are conserved across species making it easier to extrapolate findings in animals to humans. Thirdly, because of the existence of "tissue specific" microRNAs providing information about which tissue might be damaged. Measurement of these microRNAs might be taken along to assess the target organ. Obviously, in this example microRNAs are used as biomarkers of effect, such as plasma miR-122 as a marker of liver injury. Moreover, these microRNAs might be used as a panel of different microRNAs to obtain knowledge of the presence of an adverse effect. Additionally, proteins of interest might also (still) be part of this biomarker panel as demonstrated recently (Llewellyn et al. 2021).

While mRNA expression profiling has been applied more routinely in toxicogenomics-based mechanistic understanding of toxic responses, this has so far been limited for microRNA profiling. Our systematic analysis of the current literature has identified several considerations that can explain the variety of responses: i) use of different *in vitro* test systems with different degrees of differentiation status and involving both primary cells and cell lines; ii) different target organs assessed for miRNA expression;

iii) different time points were considered for evaluation of microRNA expression; iv) different concentration or dosing regimens applied in the various studies; v) lack of anchoring microRNA changes to adverse endpoints.

To make further progress on the application of microRNA profiling in mechanistic toxicology we have the following recommendations. Firstly, there is a need for more detailed concentration- time course data of microRNA expression after chemical exposure. This information will provide detailed insight in the consistency of microRNA changes over time. Secondly, studies should include diverse sets of chemicals with similar mode-of-action. This could include sets of compounds that impact on e.g. mitochondrial respiratory chain, DNA damage, oxidative stress, unfolded protein response, cytoskeletal damage, cyclin dependent kinase, etc. Thirdly, by integrating such larger miRNA expression datasets from the same test system, microRNA co-expression networks could be defined to provide learning on co-regulation of microRNA. When paralleled with mRNA transcriptional datasets, this information could then improve the integration of microRNA and mRNA data based on their co-expression (Callegaro et al. 2021). Fourthly, such studies should preferably be performed in highly differentiated human test systems representing the critical target organs for toxicity such as liver, kidney, heart and the neuronal systems. To increase the cost effectiveness of these studies, we propose the implementation of targeted microRNA sequencing approaches (Yeakley et al. 2017) limiting the overall costs for sequencing. Ultimately, identification of miRNAs that provide information on mode-of-action might be included in a plasma miRNA biomarker panel. Together, these suggested avenues for toxicological microRNA research will pave the way for microRNAs to be widely used as mechanistic biomarkers of chemical exposure as well as disease.

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