NIH Circulating Nucleic Acid/Liquid Biopsy Special Interest Group
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miRror, miRror: the biofluid-based biomarker reflective of biological effect

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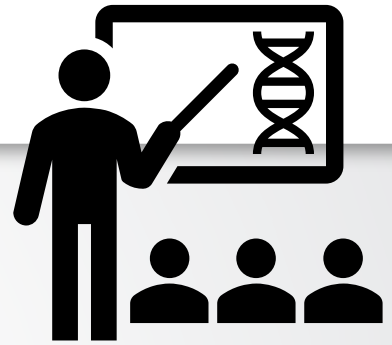
Presentation outline



1. Regulatory science drivers for biomarker development
   a) Ideal characteristics of a biomarker of effect and microRNA

2. MicroRNA case studies in tissue and biofluids
   a) Dose-responsive biomarkers of chemical mode-of-action
   b) Identification of chemical-induced tissue injury in rat urine
   c) Indicators of liver disease in an PCB-exposed residential cohort

3. Needed development of microRNA biomarkers and the road ahead

Biomarker – broad definition

An indicator signaling an event or condition in a biological system or sample and giving a measure of ***exposure***, ***effect***, or ***susceptibility***.

Such an indicator may be a ***measurable*** chemical, biochemical, physiological, behavioral, or other alteration within an organism.

(OECD, Collection of Working Definitions 2012; US National Academy of Sciences report, US NRC, 1989b; WHO International Programme on Chemical Safety, Biomarkers and Risk Assessment: Concepts and Principles 1993)

Biomarkers in toxicology

- **Biomarkers of exposure**
  - assess the amount of a chemical that is present within the body
- ***Biomarkers of effect***
  - indicators of a change in biologic function in response to a chemical exposure
- **Biomarkers of susceptibility**
  - factors that may make certain individuals more sensitive to chemical exposure

https://www.epa.gov/pesticide-science-and-assessing-pesticide-risks/defining-pesticide-biomarkers#table2

Drivers for biomarker development and use in toxicology

Higher-throughput, lower cost, and human-relevant toxicity testing

- National Research Council (NRC) - *Toxicity Testing in the 21st Century*
- European Commission - *Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH)*
- US Environmental Protection Agency (US EPA) – *The Next Generation Blueprint for Computational Toxicology*

Predictive toxicology in risk assessment, identification of vulnerable populations

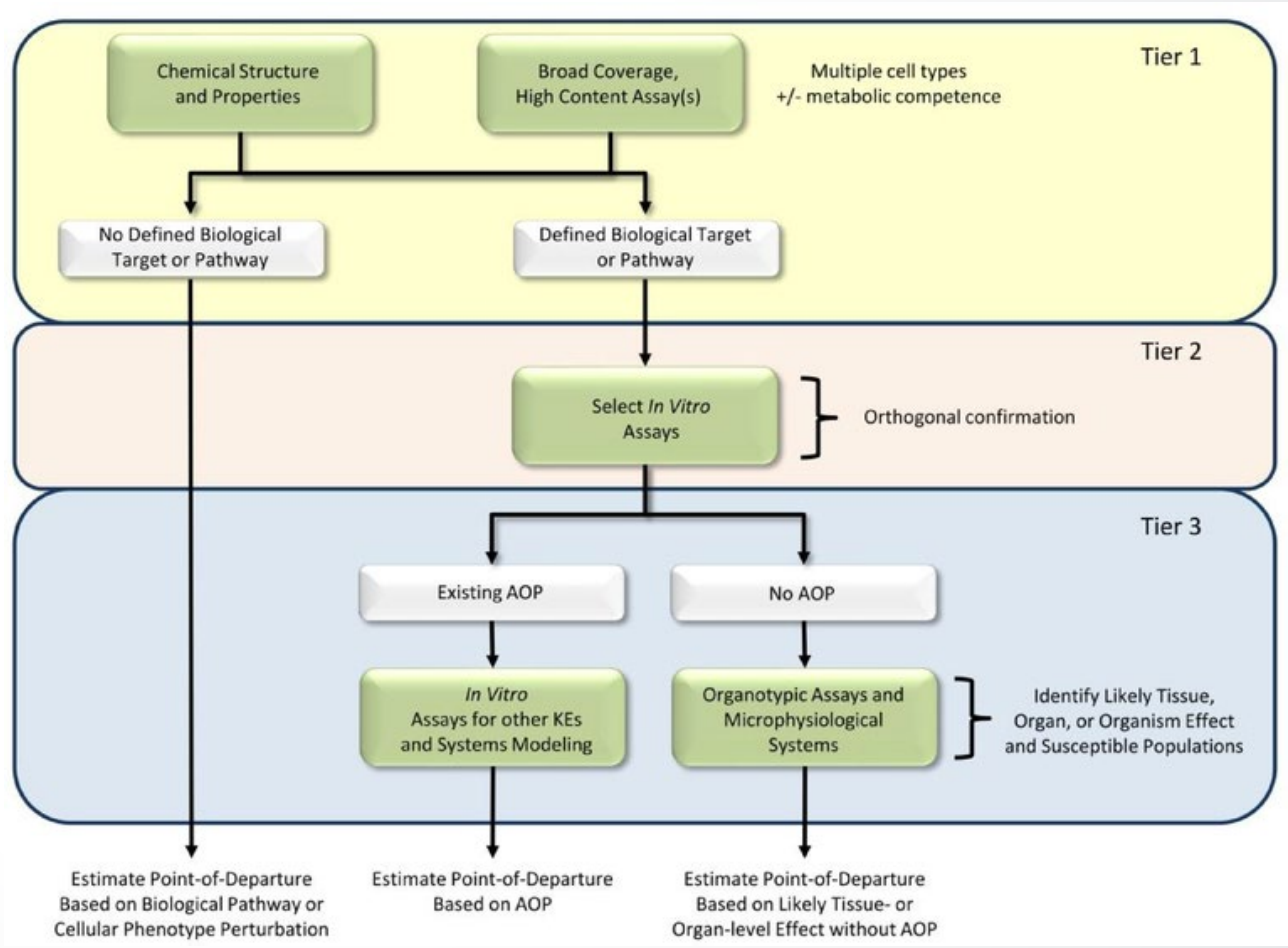
- US EPA - *Frank R. Lautenberg Chemical Safety Act*
- NRC – *Applications of Toxicogenomic Technologies to Predictive Toxicology and Risk Assessment*

Systems-level integration

- National Academy of Sciences (NAS) **–** *Use of Emerging Science for Environmental Health Decisions*

Contexts of use: Hazard identification

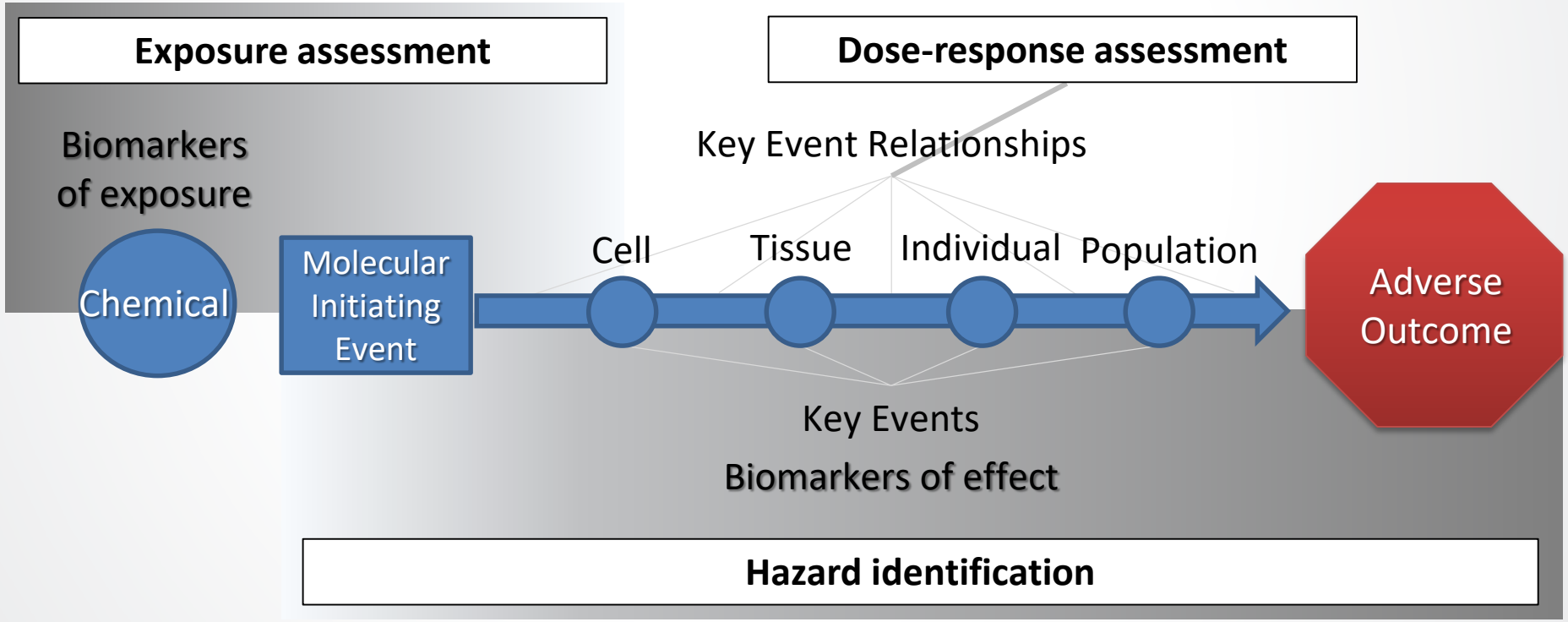
- **"The Next Generation Blueprint of Computational Toxicology at the U.S. Environmental Protection Agency"**
- High-throughput predictive endpoint measurements for thousands of assays
- Current limitations to connect molecular level alterations to more apical adverse outcomes
- Biomarkers of effect are integral for all 3 tiers of testing



Thomas et al. *Tox Sci* 2019

Regulatory context considerations

Determining risks to human health due to chemical exposure
Integration of biomarkers

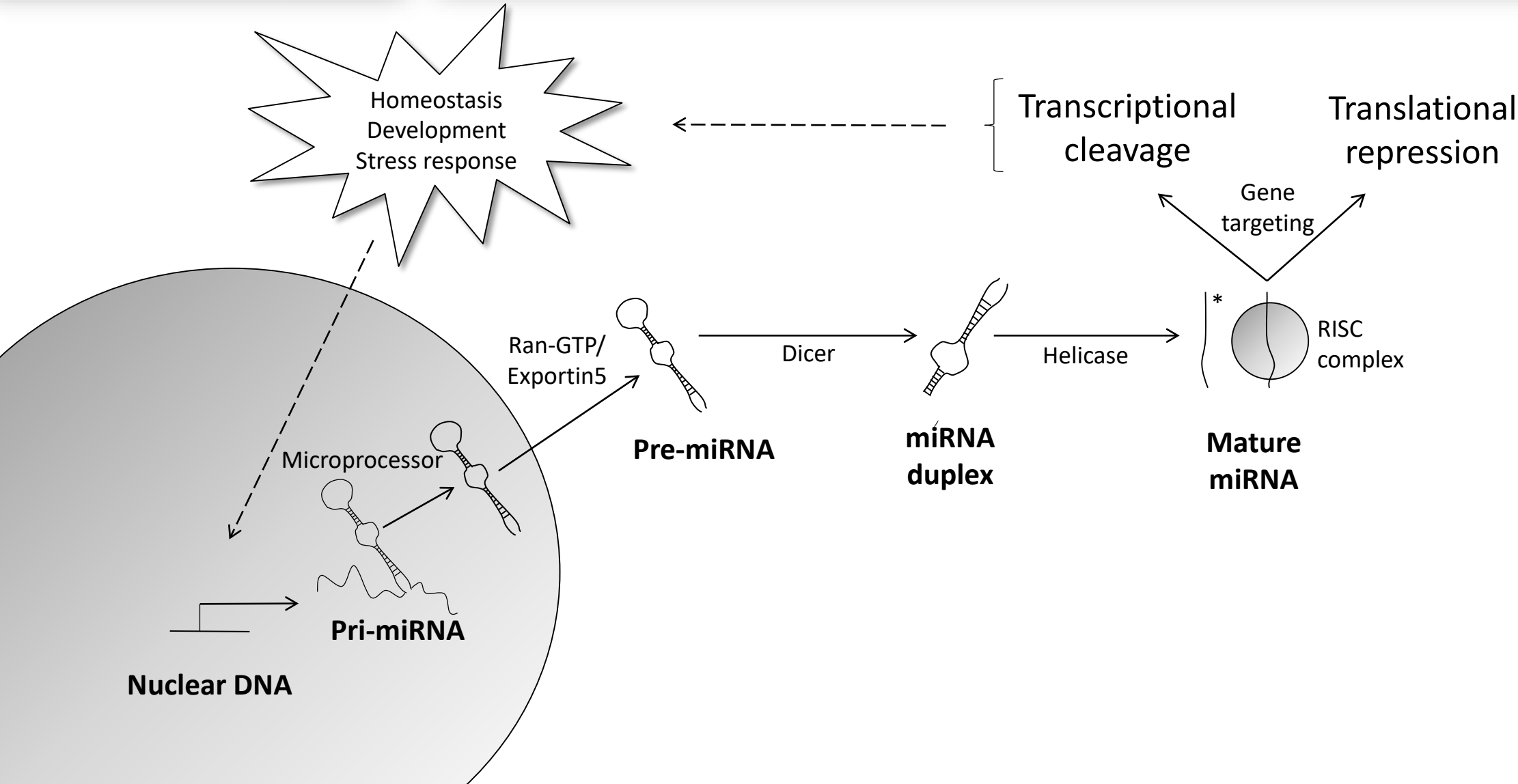


Characteristics of an ideal biomarker

Highly specific biomarker = low false positive rate	←	Specific
		Sensitive → Highly sensitive biomarker = low false negative rate
Early indication of adverse outcome	←	Predictive
		Robust → Rapid, simple, accurate, inexpensive Large measurement range
Bridge model system to human condition	←	Translatable
		Non-invasive → Present in accessible matrices

modified Table 1 from Etheridge et al., 2011 *Mut Res* PMID: 21402084

MicroRNAs as biomarkers in toxicology



Exposure and microRNA response

MicroRNAs are responsive to exogenous exposures

- Rapid response, quantitative
- Adaptive and adverse cellular responsiveness
- Exposures often linked to microRNA changes *in vivo* and *in vitro*

Examples

- *In vitro*: bisphenol A, dichlorodiphenyltrichloroethane (DDT), arsenic, polycyclic aromatic hydrocarbon (PAH)
- *In vivo (animal models)*: 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), formaldehyde, hexahydro-1,3,5-trinitro-s-triazine (RDX), perfluorooctanoic acid (PFOA)
- *In vivo (human/epi)*: polychlorinated biphenyls (PCBs), aluminum, arsenic, PAH

See review from *Environ Health Perspect 123*: 399-411, 2015

MicroRNAs in Biofluids
Non-invasive biomarkers

Predictive and non-invasive

- Passive secretion of microRNA
  - Associated with cell death and toxicity
- Active secretion of microRNA
  - Potentially vesicle-associated and involved in cell-to-cell signaling

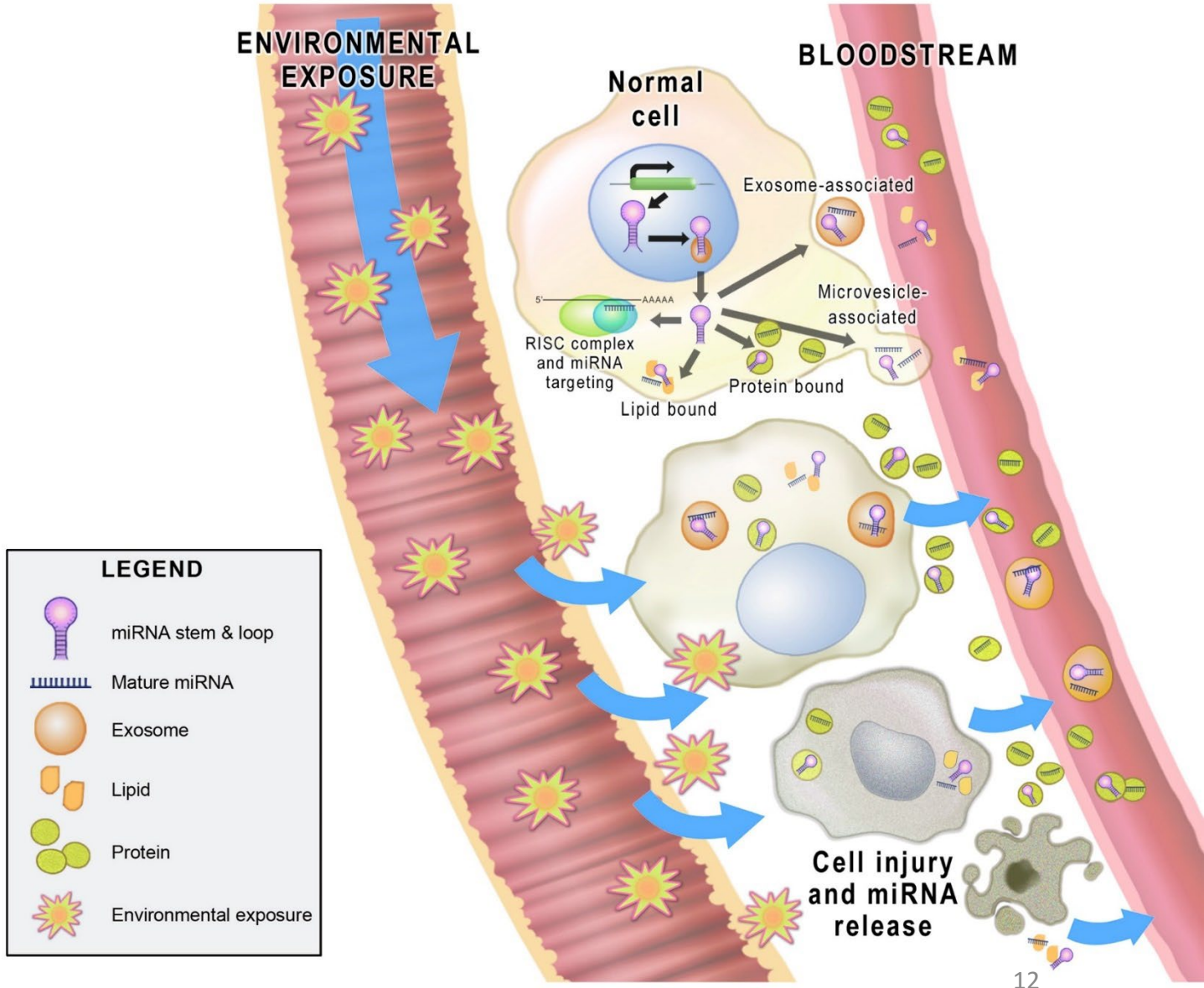


Figure 1 from *Toxicological Sciences* 152(2):264-272, 2016

Case Study #1

Early mouse liver microRNA signature due to tumorigenic exposure

Tumorigenic Phthalate Exposure in Mice

Hypothesis: Dose-responsive microRNAs correlates with gene expression and toxicology data in a PPARα mouse model of liver tumorigenesis

- Use microRNA profiling after short-term exposure of liver tumorigen

Phthalate potency predicted using transcriptional measurements

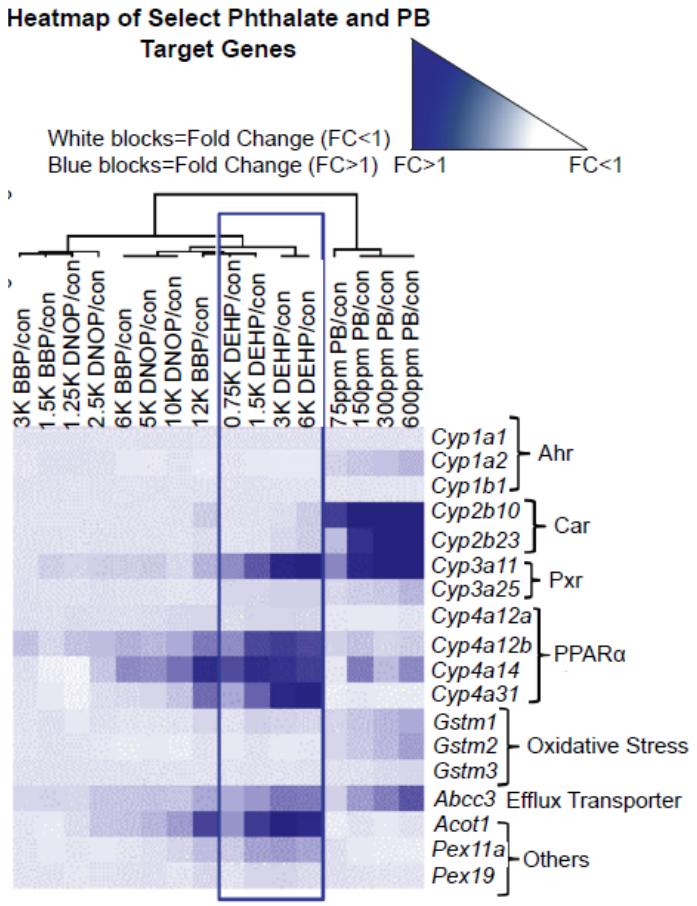


Table 3. Transcriptional (BMD_T) and apical (BMD_A) Benchmark Dose Estimates for DEHP DNOP and BBP Exposed Mouse livers.

Illumina Array Genes	DEHP (mg/kg-day) BMD_T	DNOP (mg/kg-day) BMD_T	BBP (mg/kg-day) BMD_T
Acot1	29	370	676
Cyp4a12b	61	64	210
Cyp4a31	57	912	851
Pdk4	150	>958	>1140
Abcc3	35	164	322
qPCR Genes	**DEHP (mg/kg-day) BMD_A**	**DNOP (mg/kg-day) BMD_A**	**BBP (mg/kg-day) BMD_A**
Abcc3	18	NA	NA
Acot1	77	NA	NA
Cyp4a12b	69	NA	NA
Cyp4a31	47	NA	NA
Pdk4	183	NA	NA
Functional Non-Genomic Markers	**DEHP (mg/kg-day) BMD_A**	**DNOP (mg/kg-day) BMD_A**	**BBP (mg/kg-day) BMD_A**
Ki67 (small cells only)	215	>958	>1140
PROD	42	>958	>1140
BROD	532	>958	892
Relative Liver weight	48	NA	311
Hepatocyte cytoplasmic alteration	116	NA	996
2-year Tumorigenic Potency	**DEHP (mg/kg-day) BMD_A**	**DNOP (mg/kg-day) BMD_A**	**BBP (mg/kg-day) BMD_A**
HCC	71	>1268	>1600
HCA+HCC	35	431	>1600

Potency Rank

DEHP>DNOP>BBP

DEHP>BBP>DNOP

DEHP>DNOP>BBP

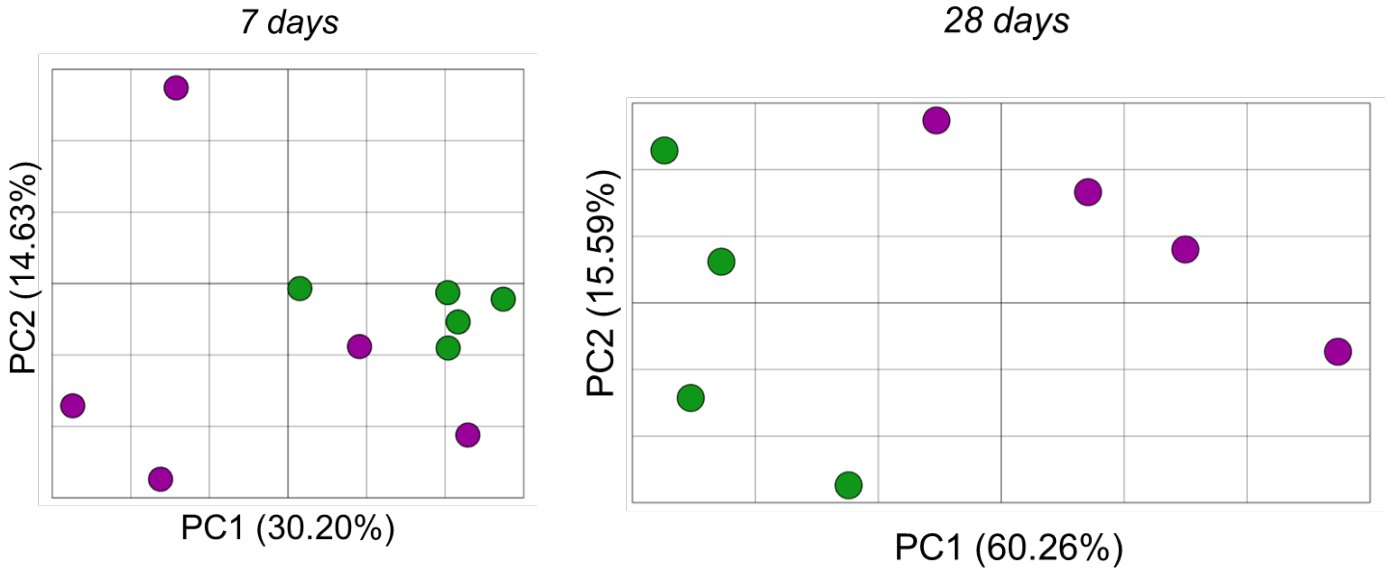
transcript profile predicts chemical potency for hepatocellular carcinoma

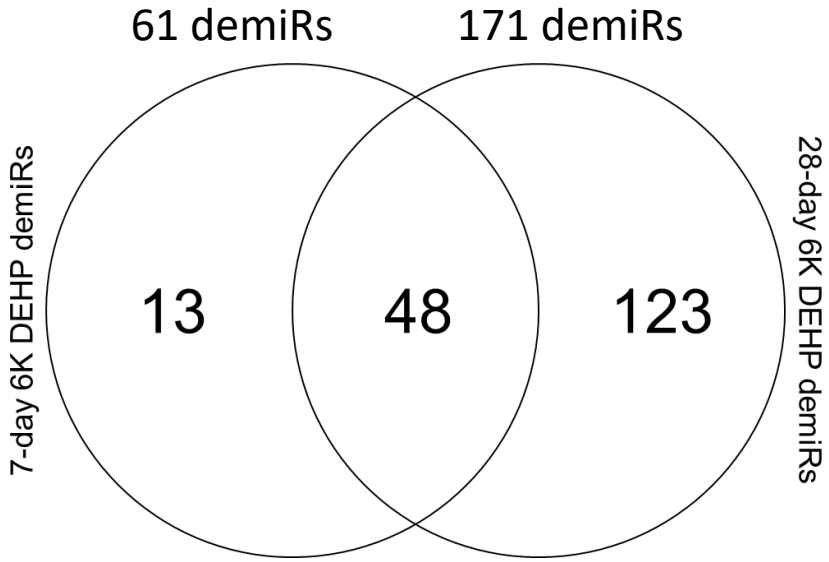
Toxicol Sci. 2016 Feb;149(2):312-25.

MicroRNA are also responsive to DEHP

Sequencing of liver RNA of 7 and 28-day DEHP treated mice

- Separation by PCA plot of liver miRNA expression
- Shared and unique miRNAs after 7 and 28 days





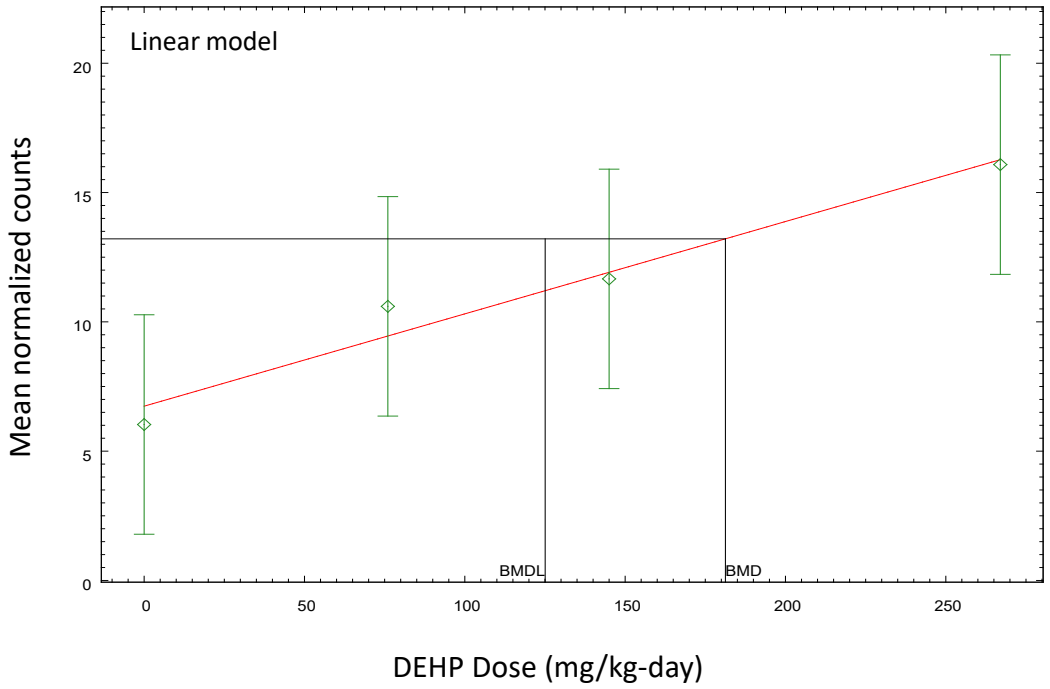
Evidence of persistent miRNA changes

Chorley et al. *Toxicol Rep*. 2020 Jun 23;7:805-815.

PPARα-linked miRNA are dose-responsive

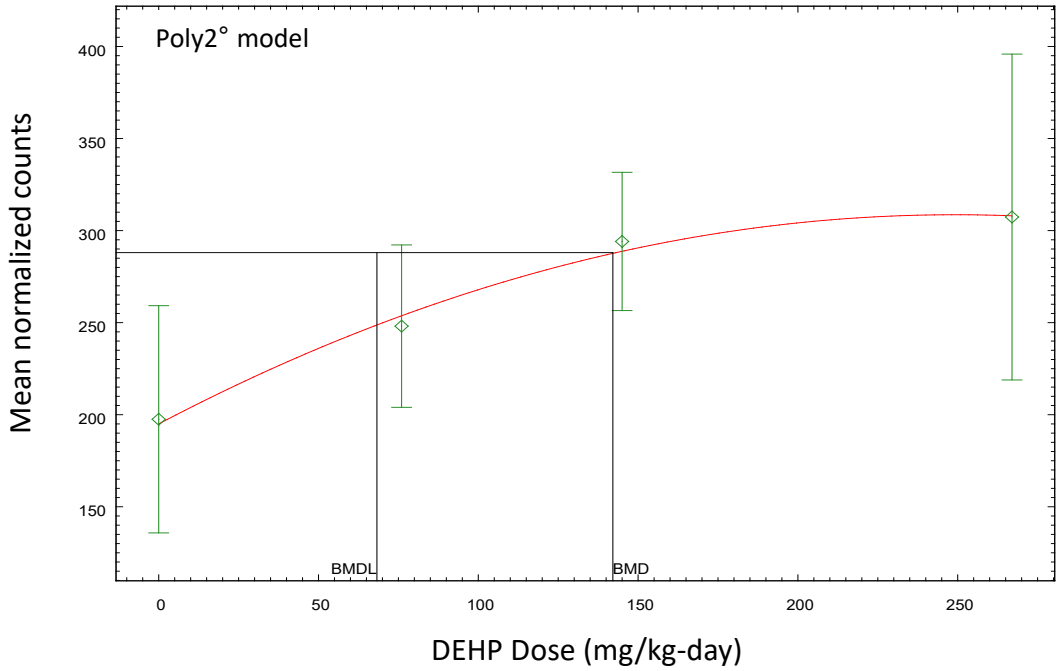
BMD analysis of dose-responsive miRNA after 7 days

mmu-miR-182-5p



BMD_{miR} = 181mg/kg-day

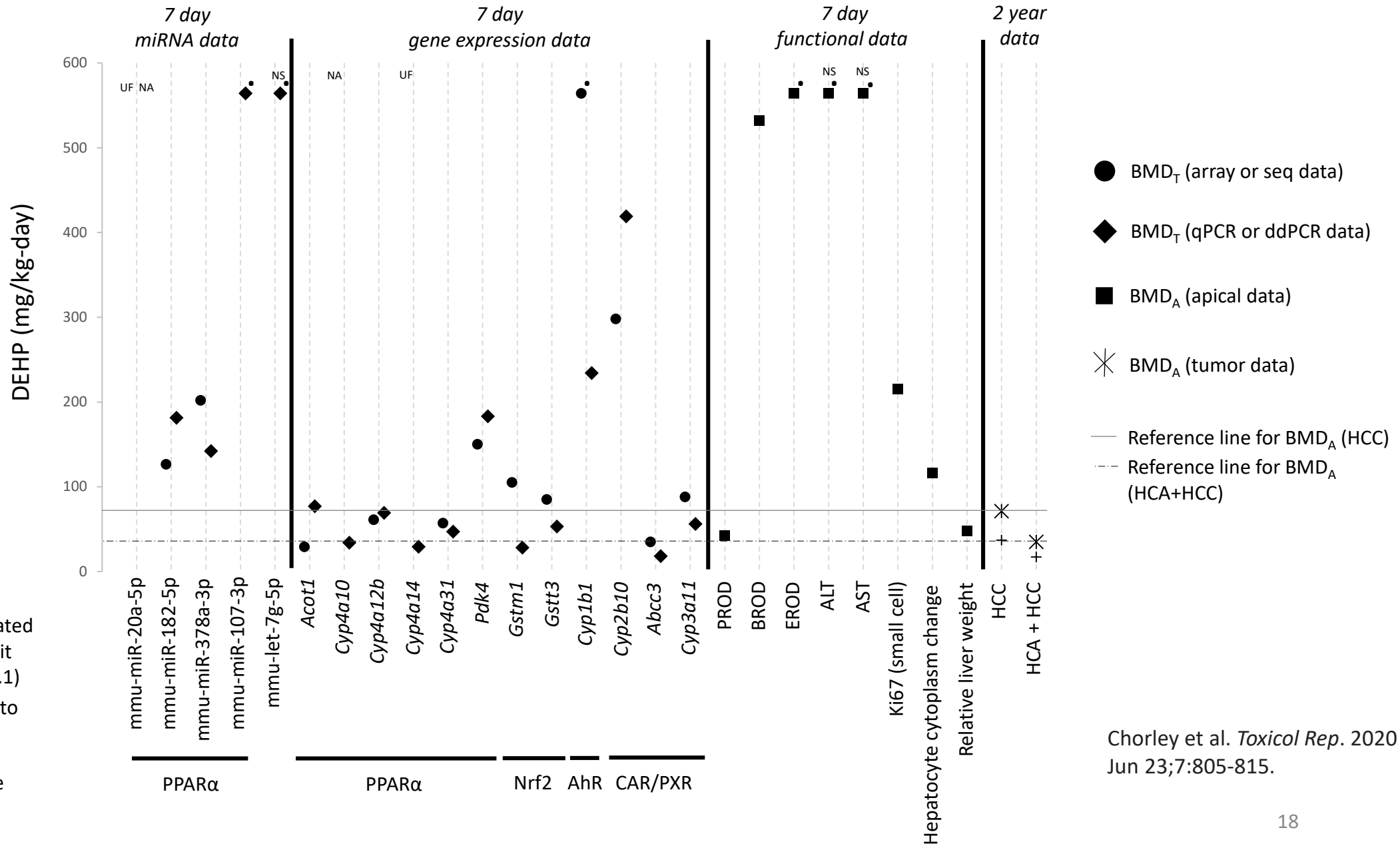
mmu-miR-378a-3p



BMD_{miR} = 142mg/kg-day

Chorley et al. *Toxicol Rep*. 2020 Jun 23;7:805-815.

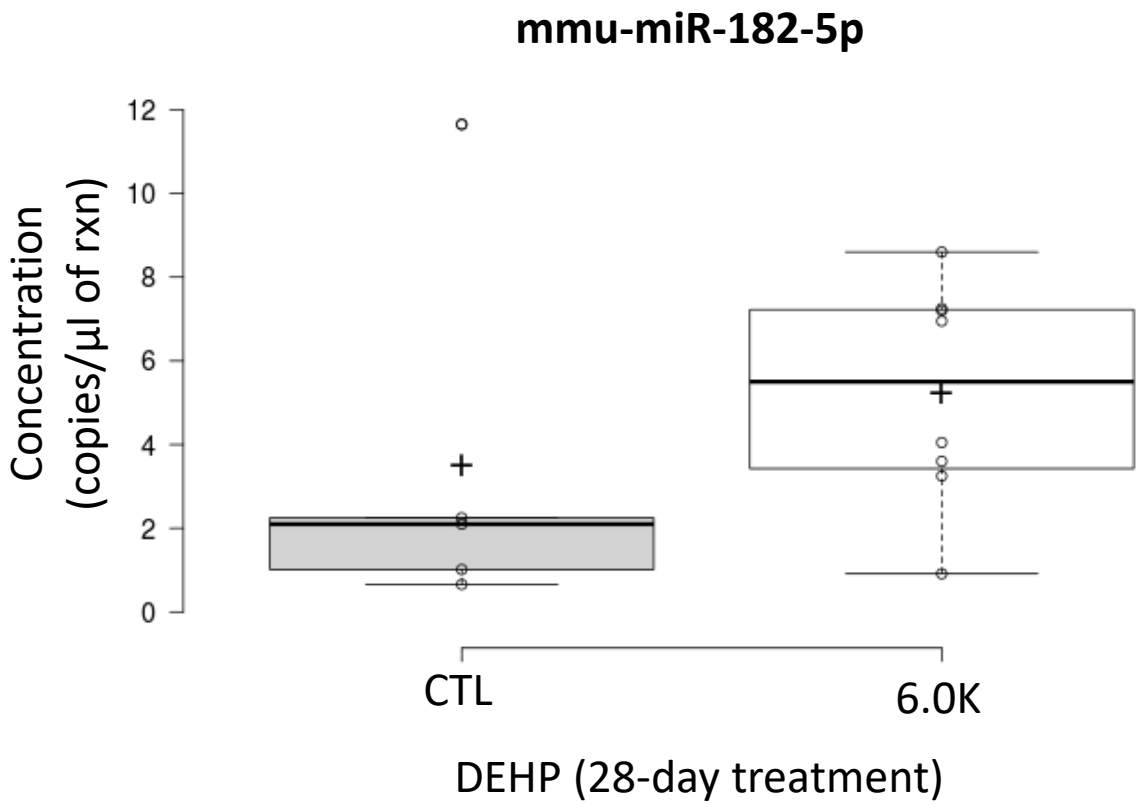
microRNAs Correlate with Gene Expression and Functional Data

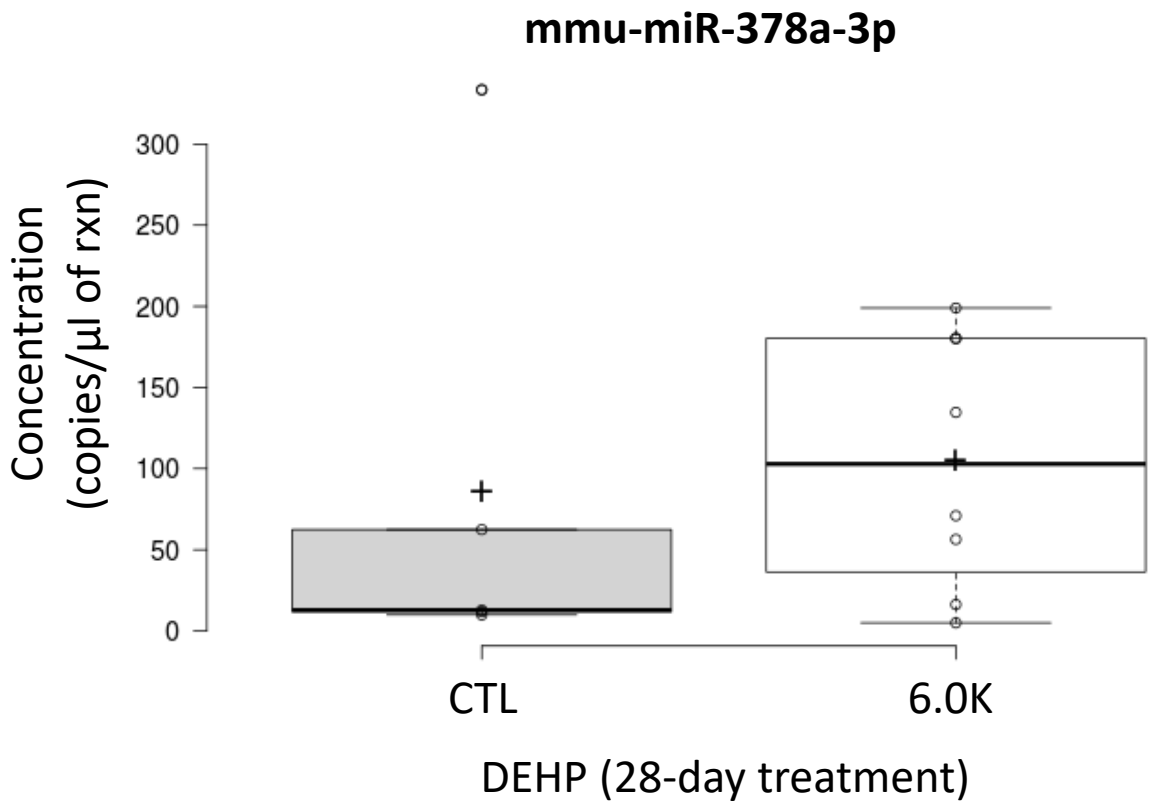


- • BMD greater than than top dose tested
- UF the response was dose-related but was an Unacceptable Fit (global goodness of fit p<0.1)
- NS not significance compared to control values for any dose tested
- NA measurement not available

Chorley et al. *Toxicol Rep*. 2020 Jun 23;7:805-815.

Candidate microRNA in serum





Chorley et al. *Toxicol Rep*. 2020 Jun 23;7:805-815.

Summary: Case Study #1

- Short-term miRNA expression demonstrates potential as a predictor for long-term outcome
- In this case study, dose-responsive miRNA are linked to the known primary mechanism of action (PPARα)
- BMD values slightly higher than PPARα-linked mRNA but better performers than many apical measurements
- Indications these miRNAs may be leaked/transfer into circulation

Case Study #2

Identifying nephron-specific miRNA biomarkers for renal toxicity

Nephrotoxicity miRNA biomarker development

Hypothesis: Urine based microRNAs a re reflective of regional damage in the kidney due to chemical exposure.

- Use meta-analysis and laser capture microdissection to identify microRNA candidates

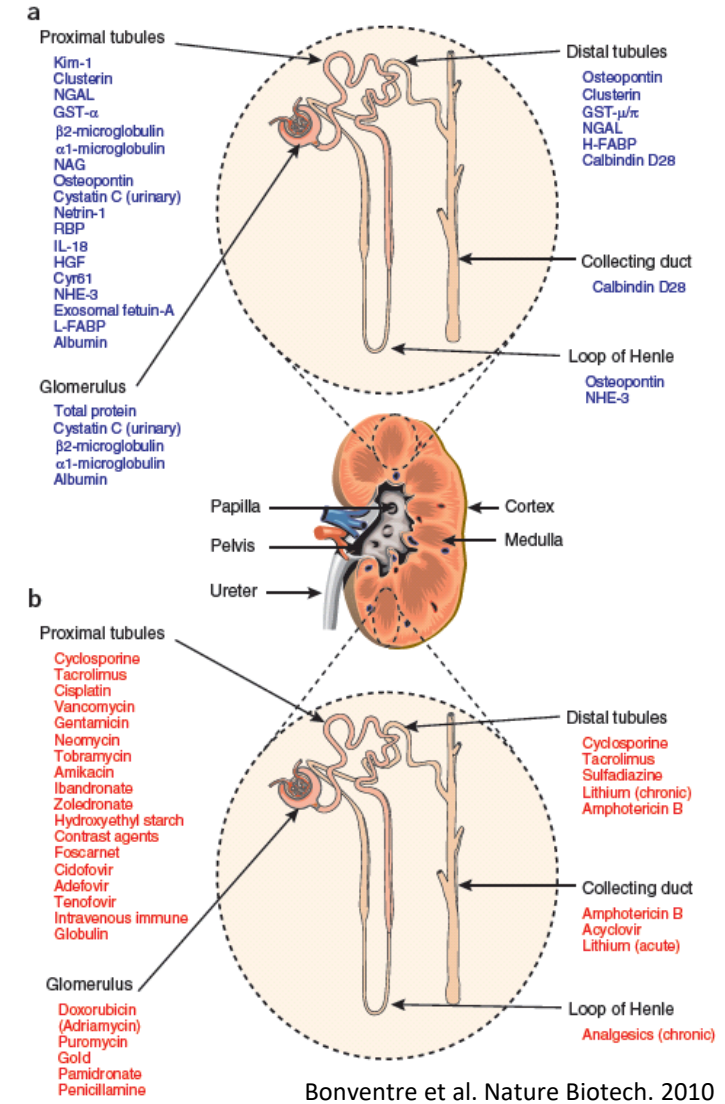
Need for kidney microRNA biomarker development

- HESI is a tripartite nonprofit that facilitates research to protect and preserve human health. The Emerging Systems Toxicology for Assessment of Risk Committee took recently completed a renal miRNA biomarker study.
- ***From a drug development perspective***: Candidate compounds can cause histopathological lesions in the kidney in animals at doses and times without changes in serum creatinine (sCr) and/or blood urea nitrogen (BUN)
- Need for kidney biomarkers that could inform the underlying pathology ("liquid biopsy")

The Health and Environmental Sciences Institute (HESI)

- ***From regulatory/EPA perspective***: Can new urine (accessible) biomarkers indicate renal toxicity due to environmental exposure (e.g. biosurveillence, weight-of evidence)?
- Can these biomarkers be linked to mechanistic perturbations in the tissue of exposure?

- **A challenge for protein biomarkers is translatability, stability, and lack of "unique" expression in discrete nephron regions**

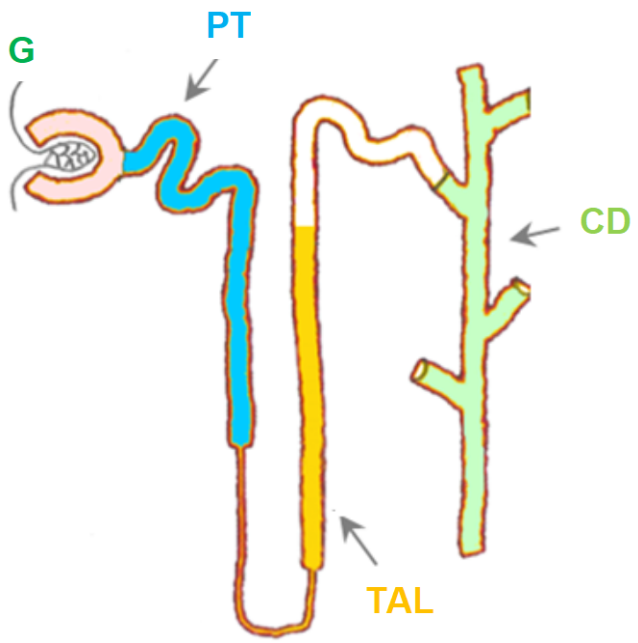


Bonventre et al. Nature Biotech. 2010

Figure 1 The utility of biomarkers to detect injury to specific nephron segments affected by various nephrotoxicants. (a) Nephron segment-specific biomarkers of kidney injury. (b) Drugs that elicit site-specific toxicity in the kidney[12,13].

Meta-analyses and confirmatory studies

Rat studies with nephron segment selective toxicants



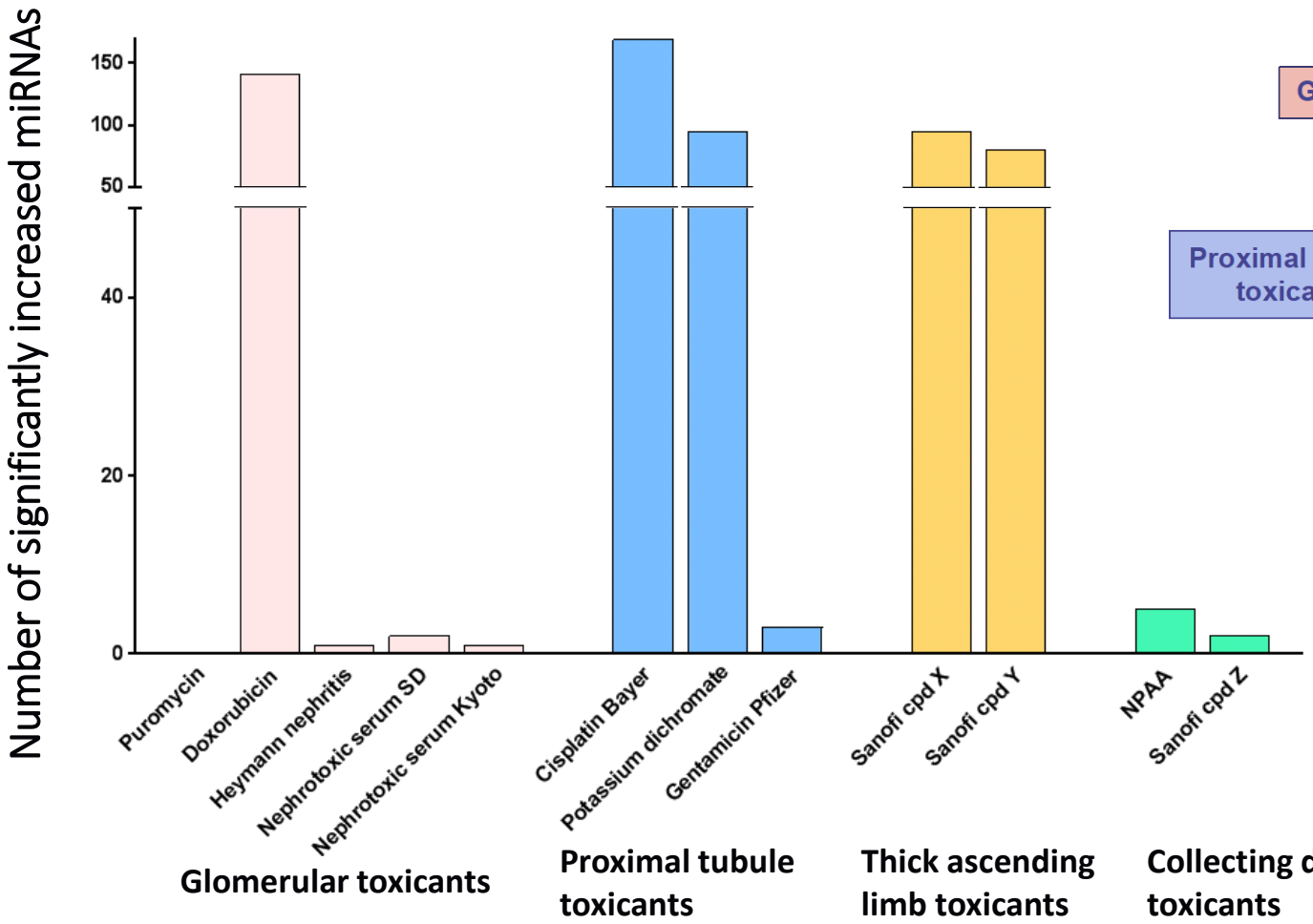
G = glomerulus; PT = proximal tubule; TAL = thick ascending limb of the loop of Henle; CD = collecting duct.

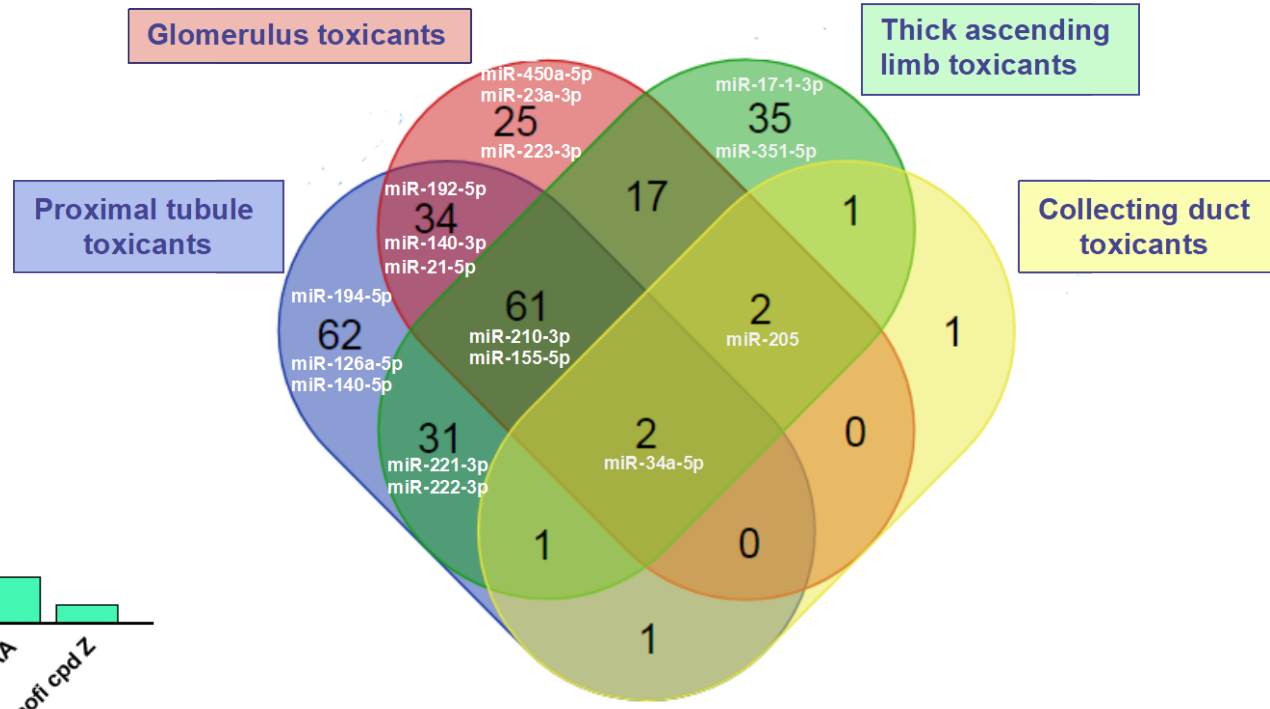
Chorley, Ellinger-Ziegelbauer, Tackett...Gautier. 2020. *In journal review.*

miRNAs in urine associated with specific renal pathologies

Site of Site injury	Compounds	Study allocation	Rat strain	Study design (dose, day of necropsy, number of animals per group)	Key histopathological findings in kidney	Main urinary protein biomarker changes*	References
Glomeruli	Puromycin	Pfizer	SD	0, 150 mg/kg – Days 2, 3, 6 N = 10	Glomerular alterations, grades 1-5	Microalbumin	Nassirpour et al 2015
	Heymann Nephritis	Pfizer	SD	0, 1 ml/200 g body weight - Days 3, 6, 9, 16 N = 10	Glomerular alterations, grades 2-3	Microalbumin*	Nassirpour et al 2015
	Doxorubicin	Janssen	SD	0, 5 mg/kg – Days 8, 15 N = 6	Glomerular alterations, grade 2 Tubular dilatation, cortico-medullary junction, grades 2-3	Microalbumin, Kim-1, NGAL	Church et al 2014
	Nephrotoxic serum	Bayer	SD	0, 5 ml/kg – Days 8, 14 N = 5 or 6	Glomerular alterations, grade 1-3 Tubular regeneration, cortex, grades 1-4	Microalbumin, Kim-1, NGAL, clusterin	Pavkovic et al 2015
			Wistar Kyoto	0, 1, 2.5, 5 ml/kg – Day 14 N = 3 or 5	Glomerular alterations, grade 1-3 Tubular regeneration, cortex, grades 1-3	Microalbumin, Kim-1, NGAL, clusterin	Pavkovic et al 2015
Proximal tubules	Potassium dichromate	Sanofi	SD	0, 5, 15 mg/kg – Day 2 N = 10	Tubular deg/necrosis, PCT (s1-2), grades 1-3	Microalbumin, NAG*	NA
	Cisplatin	Bayer	Han Wistar	0, 1, 3 mg/kg – Days 3, 5, 8, 26 N = 6	Tubular deg/necrosis, Thick descending tubule (s3), grades 1-5	Microalbumin, Kim-1, NGAL, clusterin	Pavkovic et al. 2014
	Gentamicin	Pfizer	SD	0, 50 mg/kg/day – Day 7 N = 10	Tubular deg/necrosis, PCT (s1-2), grades 1-3	Microalbumin, Kim-1	Nassirpour et al 2014
Thick ascending limb of the loop of Henle	Proprietary compound X	Sanofi	SD	0, 50, 200 mg/kg/day - Days 4, 11 N = 4 to 10	Tubular deg/necrosis, Thick ascending tubule, grades 1-3	Kim-1, microalbumin, osteopontin, clusterin	NA
	Proprietary compound Y	Sanofi	SD	0, 100, 400 mg/kg/day - Days 4, 7 N = 10	Tubular deg/necrosis, Thick ascending tubule, grades 1-2	Microalbumin*	NA
Collecting Ducts	NPAA	Sanofi	SD	0, 400 mg/kg/day – Days 4, 8, 15 N= 10	Collecting duct necrosis with loss of papilla tip, grades 1-2	RPA-1, clusterin	NA
	Proprietary compound Z	Sanofi	SD	0, 200 mg/kg/day – Days 4, 8, 15 N = 10	Necrosis of renal papilla, grades 1-2	RPA-1, clusterin	NA

Comparing urinary miRNA changes across studies and toxicants





Urinary miRNAs measured in rat studies using TaqMan arrays

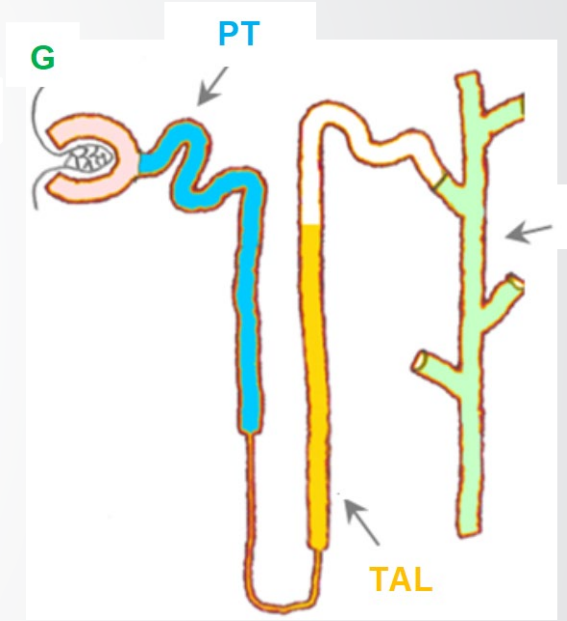
Chorley, Ellinger-Ziegelbauer, Tackett...Gautier. 2020. *In journal review.*

Indications of region specificity for some altered urine miRNA

Region-specific

Cross-region

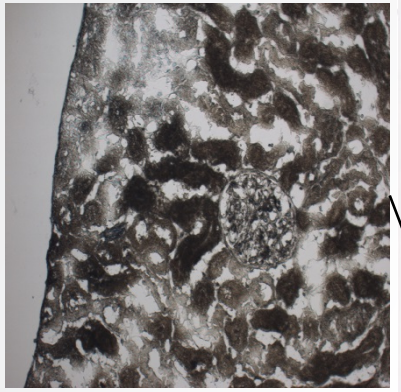
	Glomerulus toxicants	Proximal tubule toxicants		Thick ascending limb toxicants	
	Doxorubicin	Cisplatin	Potassium dichromate	Sanofi X	Sanofi Y
miR-421	-	47.9	3.3	-	-
miR-872-5p	-	17.4	2.4	-	-
miR-140-5p	-	**3.1**	**3.5**	-	-
miR-1306	-	-	-	-	29.6
miR-17-1-3p	-	-	-	**18.0**	**4.7**
miR-351-5p	-	-	-	8.2	3.7
miR-347	-	-	-	5.8	10.6
miR-222-3p	-	**23.5**	-	**38.8**	**6.3**
miR-221-3p	-	**15.5**	-	**42.8**	-
miR-210-3p	**28.7**	**650.0**	-	**39.6**	**12.8**
miR-192-5p	**5.7**	**68.3**	-	-	-
miR-184	76.1	-	-	-	-
miR-223-3p*	**41.4**	-	-	-	-
miR-369-3p	17.4	-	-	-	-
mir-9#	10.0	-	-	-	-
miR-34c-3p	**12.1**	**11.3**	-	-	-



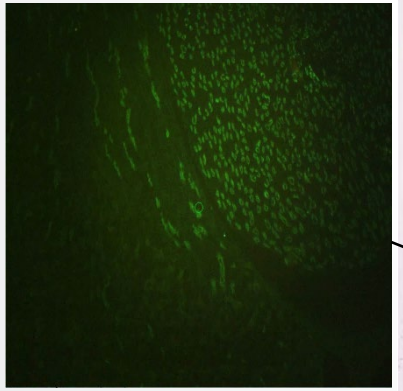
Chorley, Ellinger-Ziegelbauer, Tackett...Gautier. 2020. *In journal review.*

Fold-changes in urine versus respective controls in the original rat toxicant studies

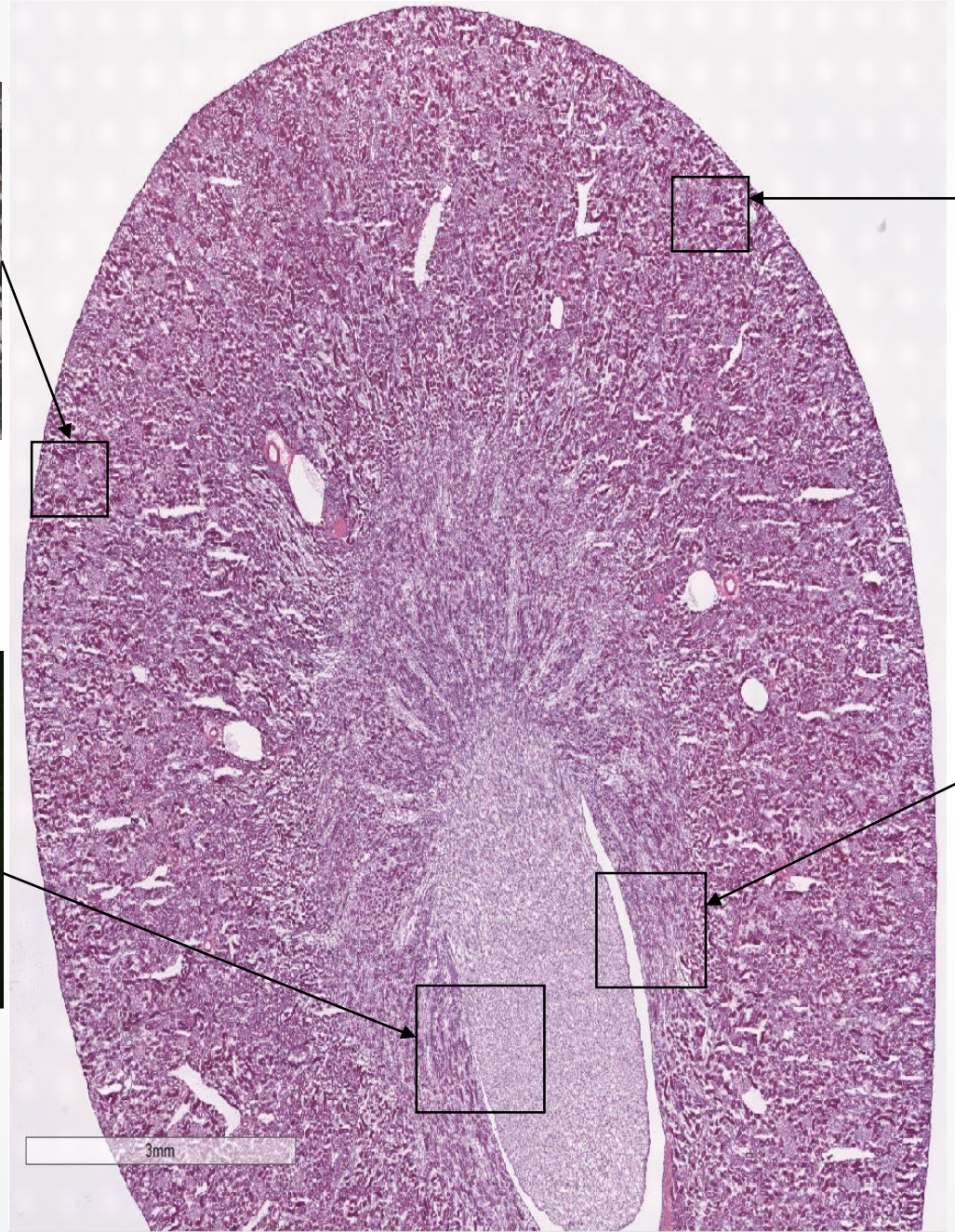
Confirm miRNA expression localization with laser-capture microdissection

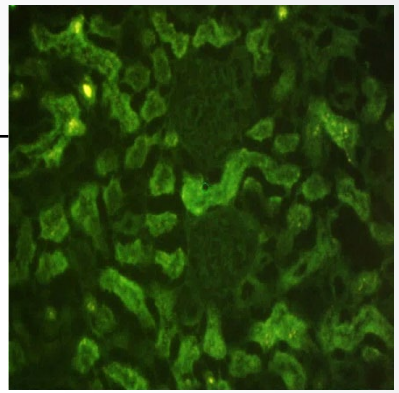


Glomerulus (Histogene)

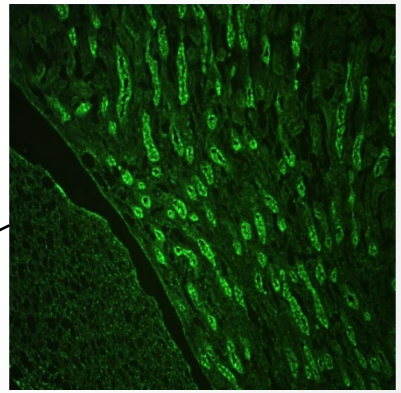


Collecting ducts (AQP-2)





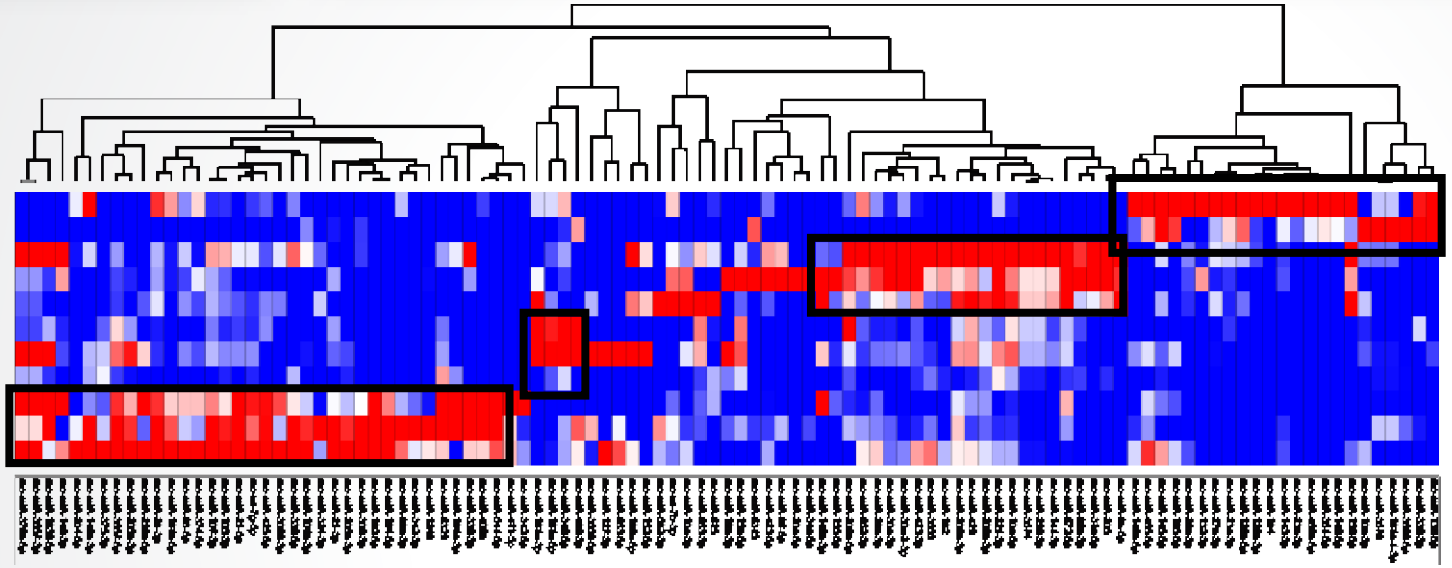
Proximal tubules (α-GST)



Thick ascending limb of the loop of Henle (THP)

- sRNA-seq
- Fireplex
- digital drop PCR

Small RNA sequencing identifies nephon-specific miRNA; validation with ddPCR/FirePlex



Toxicant response in urine ↓

		ddPCR (target/miR-30d-5p)					Fireplex (MFi)					Sequencing (Normalized counts)					Correlation		
		Glomerulus	Proximal Tubule	Collecting Duct	Thick Ascending Limb		Glomerulus	Proximal Tubule	Collecting Duct	Thick Ascending Limb		Glomerulus	Proximal Tubule	Collecting Duct	Thick Ascending Limb		Correlation ddPCR to Fireplex	Correlation ddPCR to Seq	Correlation Fireplex to Seq
G/PT	rno-mir-143-3p	18.34	9.17	6.27	9.75		298.83	194.32	142.02	134.63		53401.94	28673.15	29779.12	22349.57		0.92	0.88	0.95
PT	rno-mir-192-5p	5.23	39.50	2.05	6.17		12.44	174.48	14.11	24.29		4346.56	89910.60	7119.19	13259.77		1.00	0.99	1.00
PT/TAL	rno-mir-140-5p	0.74	1.24	0.47	0.99		5.15	2.98	1.14	1.99		-	-	-	-	*	0.19	-	-
G/PT/TAL	rno-mir-221-3p	0.06	0.09	0.33	0.47		12.53	26.00	142.06	69.66		52.96	50.99	204.15	118.16		0.67	0.69	0.99
TAL	rno-mir-210-3p	0.32	0.18	0.37	0.57		4.54	4.76	42.50	50.37		9.54	19.45	116.24	60.16		0.84	0.41	0.81
G	rno-mir-17-1-3p	0.10	0.16	0.23	0.63		2.08	3.91	1.15	7.72		-	-	-	-	*	0.86	-	-
PT/TAL	rno-mir-223-3p	2.29	1.61	1.16	2.37		0.83	0.07	0.34	0.37		-	-	-	-	*	0.54	-	-
	rno-mir-222-3p	0.29	0.40	0.91	0.99		3.75	4.05	20.20	17.43		-	-	-	-	*	0.97	-	-

Chorley, Ellinger-Ziegelbauer, Tackett, Harrill...Gautier. 2020. *In journal review.*

Summary: Case Study #2

- Combination of meta-analysis of rat studies using drug exposure of known drug-induced kidney injury and laser-capture microdissection identified candidate urine-base biomarkers of nephrotoxicity
- This indicates region-specific expression of miRNA that is released into accessible biofluids (within context)
- Adds further evidence that miRNA are specific biomarkers of toxicity that can be utilized non-invasively in toxicological studies; thereby reducing costs and increasing precision of these studies
- Are these translatable to other species? Human?

Case Study #3

Serum-based microRNA biomarkers for fatty liver disease in PCB-exposed human population

Serum microRNA associated with toxicant-associated liver disease

Hypothesis: Previously identified individuals with toxicant-associated fatty liver disease will exhibit an altered liver microRNA profile in serum.

- Use targeted panel to directly measure microRNA in archived serum and correlate with other metrics in cohort.

Anniston Community Health Survey (ACHS)

- PCB (polychorinated biphenyls) mixtures produced at a chemical plant from 1929-1971 in Anniston, Alabama
- Large, cross-sectional epidemiological study of residential population:
  - Increased PCB levels compared to NHANES reference (2-3 fold)
  - High prevalence of obesity (54%)
  - Associations between PCB exposures and hypertension, diabetes, and dyslipidemia (conditions commonly seen in metabolic syndrome)

Linda Birnbaum (NIEHS)

Pavuk et al. Sci Total Environ 2014; Goncharov et al. J Hypertension 2010; Silverstone et al. EHP 2012; Aminov et al. Env. Health 2013; Cave et al. J Occ Env Med 2011

Biomarker Evidence of TASH

- Toxicant-associated steatohepatitis (TASH) is a form of necrotic liver disease associated with both industrial and environmental chemical exposures.
- Cave *et al.* found evidence of TASH in 738 ACHS samples (phase I)
  - Fragment analyses of CK18 in serum indicate oncotic necrosis or apoptotic death processes in hepatocytes
  - Can distinguish TASH from other liver disease
  - Positive associations of steatohepatitis with elevated pro-inflammatory cytokines, insulin resistance, hypertriglyceridemia and specific PCB congeners
    - Linked to environmental liver disease

Matt Cave (U. of Louisville)

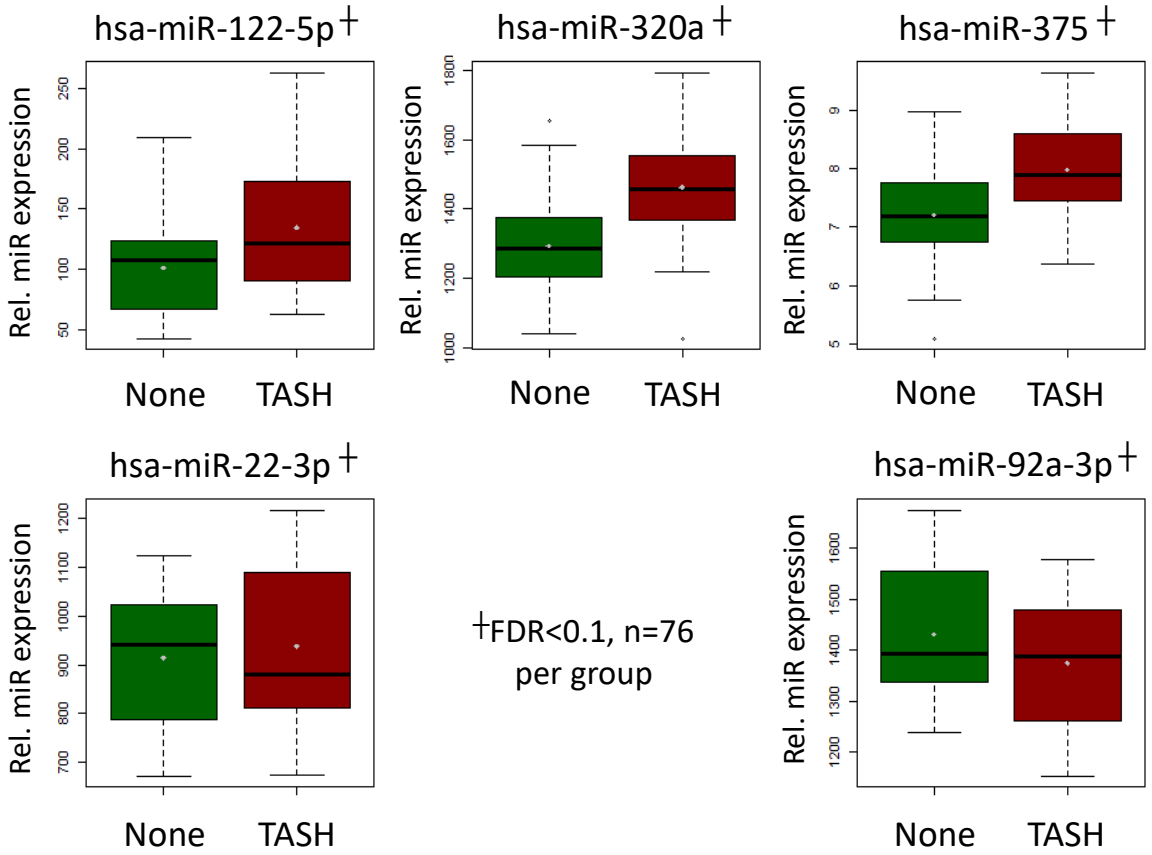
Clair HB, Pinkston CM, Rai SN, et al. *Toxicol Sci*. 2018;164(1):39-49.

Profile screen: liver-associated miRNAs

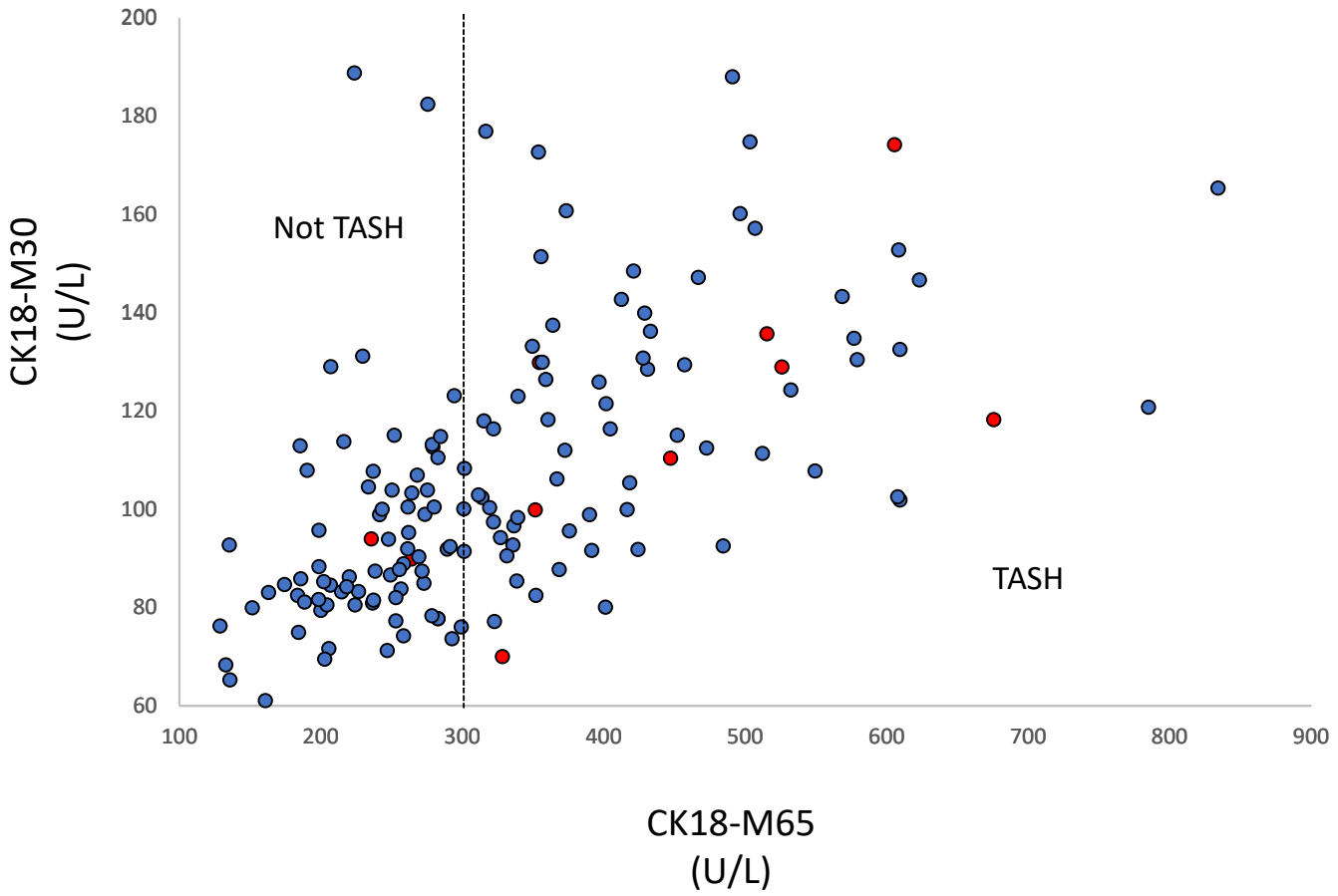
Bullets indicate location/species of altered miRs in liver disease/toxicity, based on published literature

- human serum or plasma
- human liver/hepatocytes
- mouse/rat serum or plasma
- mouse/rat liver

Significant difference in serum miRNA in TASH individuals



High miR-122 expression level correlates with TASH



hsa-miR-122-5p

- <2 X SD of mean of normalized miR in serum
- ≥2 X SD of mean of normalized miR in serum

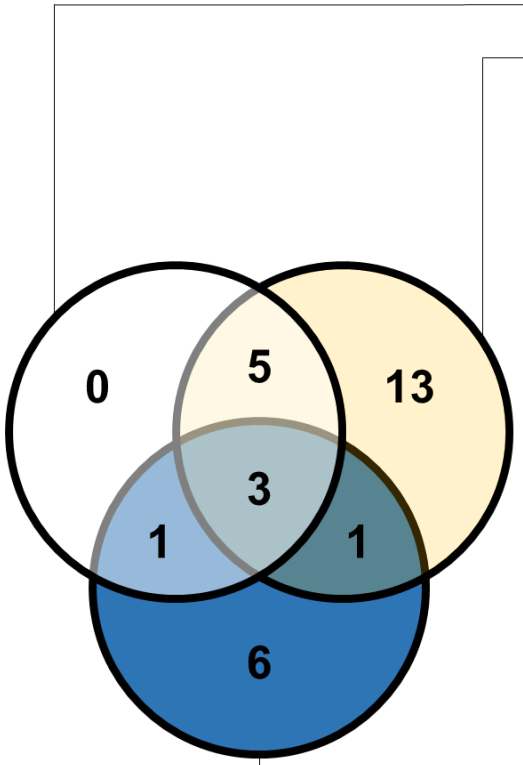
Recent follow-up: Full ACHS cohort examined (n=738)

miRNA	Differentially regulated miRNAs in Necrotic Liver Disease (n=359)			Differentially regulated miRNAs in Other Liver Disease (n=85)			Keratin 18 (n=738)			
							K18 M65		K18 M30	
	FC	FDR	P_{raw}	FC	FDR	P_{raw}	β±SE	P-value	β±SE	P-value
Up-regulated miRNA										
miR122-5p	**1.46**	**<0.0001**	**<0.0001**	**2.91**	**<0.0001**	**<0.0001**	**0.88±0.08**	**<0.0001**	**0.76±0.08**	**<0.0001**
miR192-5p	**1.20**	**0.003**	**0.0003**	**1.64**	**<0.0001**	**<0.0001**	**0.41±0.05**	**<0.0001**	**0.36±0.05**	**<0.0001**
miR320a	**1.05**	**0.15**	**0.06**	0.97	0.67	0.49	0.03±0.02	0.22	-0.01±0.02	0.55
miR99a-5p	**1.09**	**0.17**	**0.06**	**1.38**	**<0.0001**	**<0.0001**	**0.24±0.05**	**<0.0001**	**0.24±0.05**	**<0.0001**
Down-regulated miRNA										
miR24-3p	**0.92**	**0.02**	**0.003**	0.95	0.40	0.23	**-0.07±0.03**	**0.01**	-0.02±0.03	0.38
miR197-3p	**0.91**	**0.09**	**0.02**	**0.88**	**0.14**	**0.046**	**-0.11±0.04**	**0.01**	**-0.09±0.04**	**0.04**
let7d-5p	**0.94**	**0.12**	**0.03**	**0.82**	**0.0003**	**<0.0001**	**-0.15±0.03**	**<0.0001**	**-0.12±0.03**	**0.0001**
miR221-3p	**0.94**	**0.14**	**0.04**	**0.84**	**0.003**	**0.0005**	**-0.14±0.03**	**<0.0001**	**-0.12±0.03**	**0.0001**
miR17-5p	**0.96**	**0.14**	**0.049**	**0.93**	**0.13**	**0.04**	**-0.08±0.02**	**<0.0001**	-0.04±0.02	0.07

Associated with TASH in pilot samples

Associated with other related measures in pilot samples

Recent follow-up: Full ACHS cohort examined (n=738)



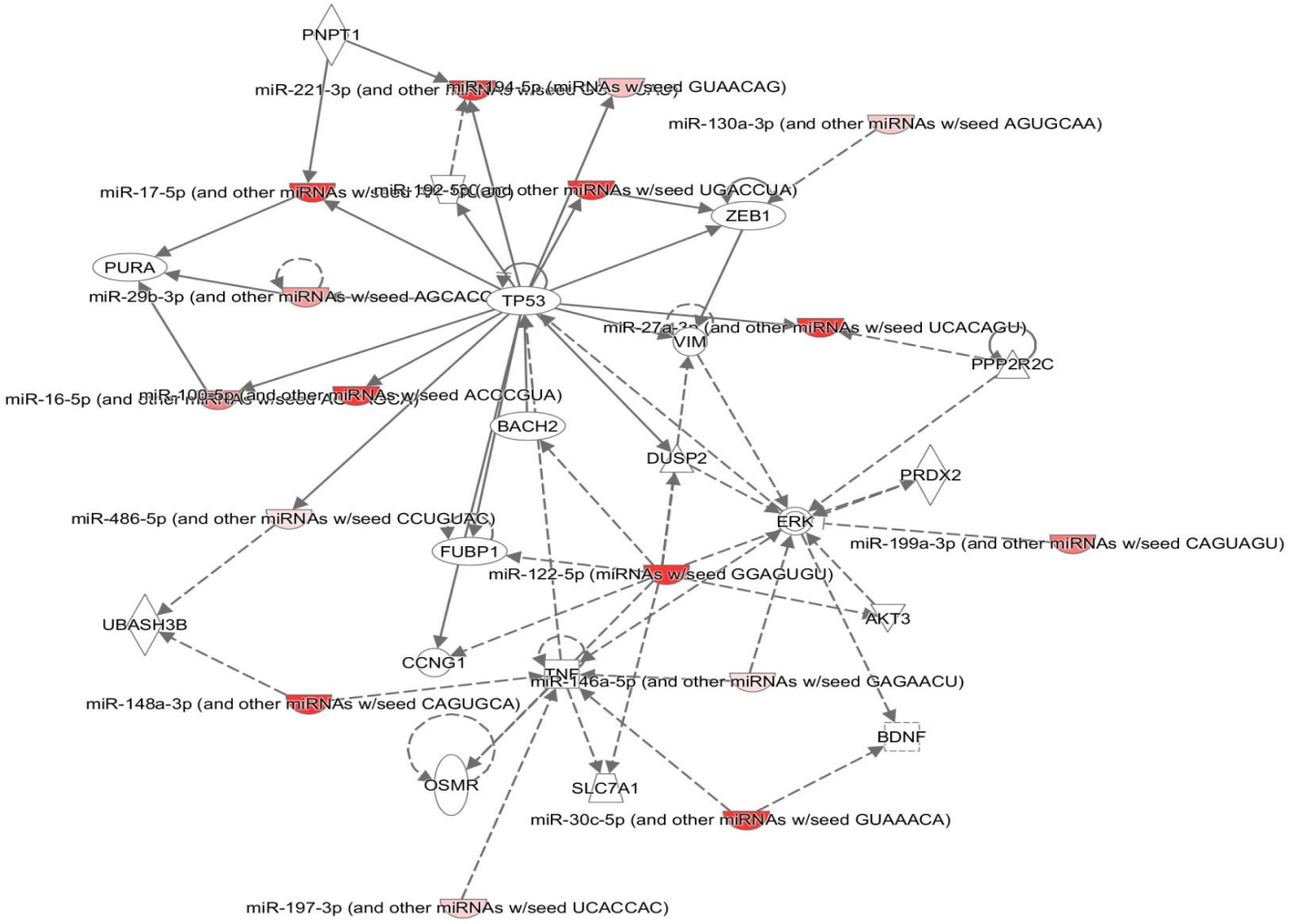
Cell death marker associated miRNAs	Liver disease associated miRNAs	PCB associated miRNAs
miR-122-5p	miR-122-5p	miR-122-5p
miR-192-5p	miR-192-5p	miR-192-5p
miR-99a-5p	miR-99a-5p	miR-99a-5p
miR-197-3p	miR-197-3p	miR-185-5p
let-7d-5p	let-7d-5p	miR-15a-5p
miR-221-3p	miR-221-3p	miR-22-3p
miR-17-5p	miR-17-5p	miR-21-5p
miR-24-3p	miR-24-3p	miR-29c-3p
let-7i-5p	miR-320a	miR-320a
miR-130a-3p		miR-130a-3p
miR-146a-5p		miR-451a
miR-148a-3p		
miR-15b-5p		
miR-181a-5p		
miR-18a-5p		
miR-194-5p		
miR-199a-3p		
miR-27b-3p		
miR-29a-3p		
miR-30c-5p		
miR-486-5p		
mmu-miR-199a-5p		

Pathway analysis of necrotic biomarker-associated miRNAs

(B) Hepatocyte death markers-associated miRNAs

Enriched tissue specific toxicity	p-value	Associated miRNAs
Renal Tissue		
Glomerular Injury	1.23E-09-1.23E-09	miR-99a-5p, miR-130a-3p, miR-15b-5p, miR-197-3p, miR-30c-5p, miR-486-5p
Renal Inflammation	1.23E-09-1.23E-09	miR-99a-5p, miR-130a-3p, miR-15b-5p, miR-197-3p, miR-30c-5p, miR-486-5p
Renal Nephritis	1.23E-09-1.23E-09	miR-99a-5p, miR-130a-3p, miR-15b-5p, miR-197-3p, miR-30c-5p, miR-486-5p
Liver Tissue		
Hepatocellular carcinoma	3.99E-15-2.44E-02	miR-99a-5p, miR-122-5p, miR-130a-3p, miR-146a-5p, miR-148a-3p, miR-15b-5p, miR-17-5p, miR-181a-5p, miR-192-5p, miR-199a-3p, miR-199a-5p, miR-221-3p, miR-27b-3p, miR-29a-3p, miR-30c-5p
Liver Hyperplasia/Hyperproliferation	3.99E-15-2.44E-02	miR-99a-5p, miR-122-5p, miR-130a-3p, miR-146a-5p, miR-148a-3p, miR-15b-5p, miR-17-5p, miR-181a-5p, miR-192-5p, miR-199a-3p, miR-199a-5p, miR-221-3p, miR-27b-3p, miR-29a-3p, miR-30c-5p
Liver Inflammation/Hepatitis	1.4E-11-1.4E-11	miR-99a-5p, miR-130a-3p, miR-15b-5p, miR-199a-5p, miR-221-3p, miR-27b-3p
Liver Cirrhosis	2.28E-08-8.65E-08	miR-99a-5p, miR-130a-3p, miR-15b-5p, miR-181a-5p, miR-199a-5p, miR-221-3p, miR-27b-3p
Cardiac Tissue		
Cardiac Dilation	8.95E-06-8.95E-06	miR-146a-5p, miR-17-5p, miR-199a-3p, miR-30c-5p, miR-486-5p
Cardiac Enlargement	8.95E-06-8.95E-06	miR-146a-5p, miR-17-5p, miR-199a-3p, miR-30c-5p, miR-486-5p
Congenital Heart Anomaly	2.82E-02-2.82E-02	miR-130a-3p

Pathway analysis of necrotic biomarker-associated miRNAs



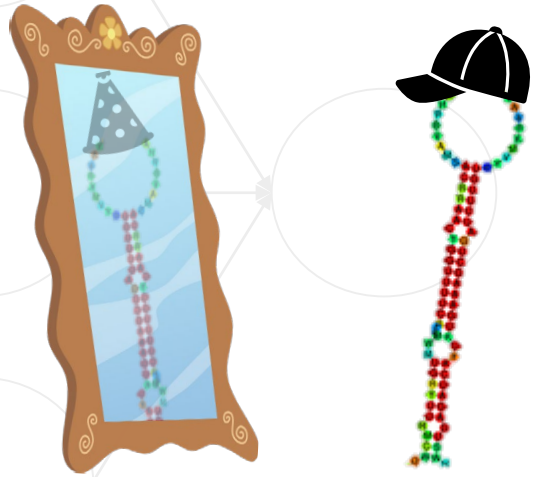
De novo pathway of necrotic biomarker associated miRNA in ACHS

Summary: Case Study #3

- MicroRNA are pathway-based and tissue-specific biomarkers that are stable in blood, urine and other biofluids
- This case study demonstrated associations between liver toxicity-linked miRNAs and PCB-mediated liver disease (based on other biomarker measurements)
- Such efforts will help develop more sensitive and specific biomarkers for clinical, industrial, and regulatory applications

Overall summary

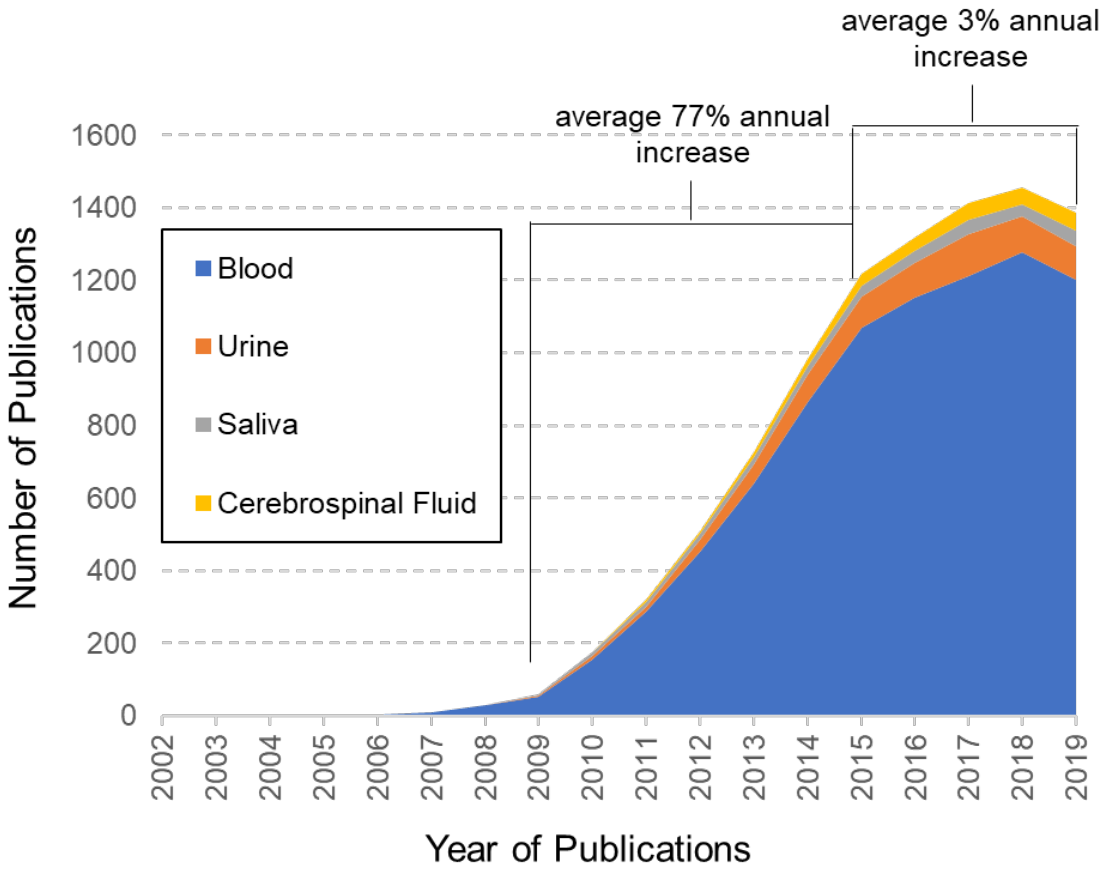
- Case studies have demonstrated:
  - microRNAs are dose-responsive to chemical perturbation *in vivo* and are linked to mode-of-action for downstream adverse outcome
  - microRNAs can be specific for regional toxicity while being detected in accessible biofluids
  - microRNAs can correlate to disease biomarkers in environmentally exposed residential cohorts

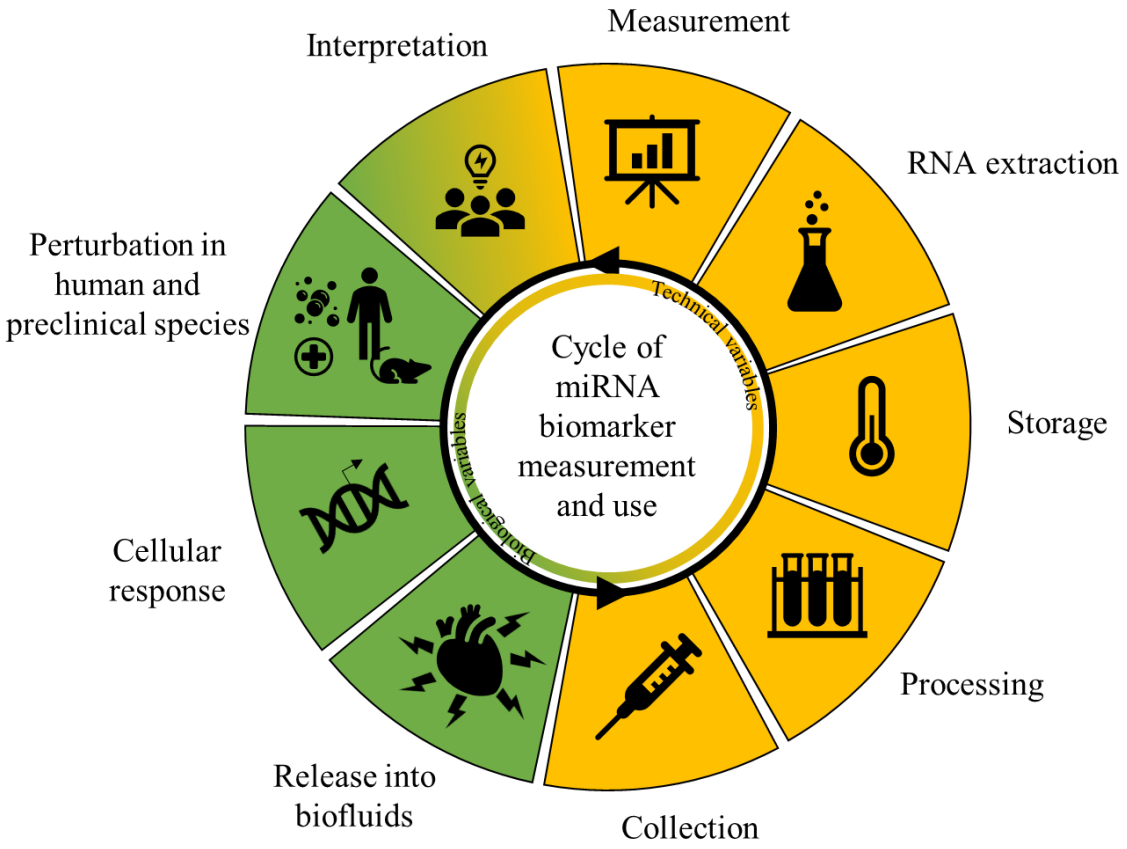


- This is significant but there are still gaps
  - Can we overcome variability associated with these measurements? Can we establish a baseline?
  - Can we more definitively link serum microRNA alterations to Key Events of Adverse Outcome?
  - Can these biomarkers be more predictive and utilized in a screening context (*in vitro, alternative models*)?

Where do we need to go from here?

There are many uncertainties associated with microRNA biomarkers





Chorley, Atabakhsh, Doran, Gautier, Ellinger-Ziegelbauer, Jackson, Sharapova et al. 2020. *In journal review.*

Acknowledgements: Case Study #1

US EPA

Charles Wood (now at Boehringer Ingelheim)

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Gleta Carswell

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NSF International, Ann Arbor, MI

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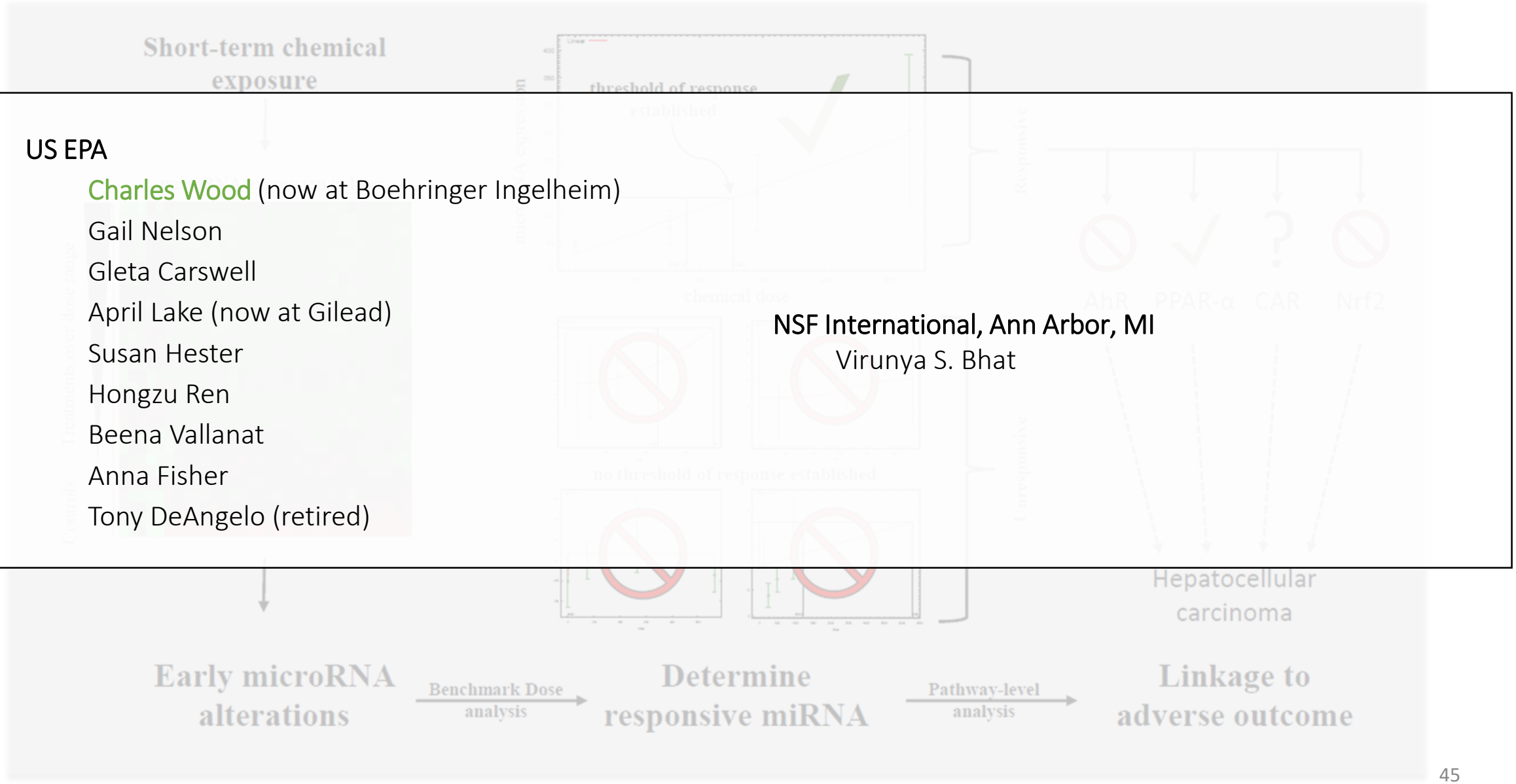

Acknowledgements: Case Study #2/Gap analysis

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HESI COMMITTEE

Emerging Systems Toxicology for the Assessment of Risk (eSTAR)

OUR MISSION

The committee's mission is to develop and deliver innovative systems toxicology approaches for risk assessment.

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2019 COMMITTEE HIGHLIGHTS

Participating Organizations
14 government/regulatory agencies, 9 academic/research institutes, 17 industry

Publications
2 accepted/published, 1 submitted, 2 in progress

Scientific Meetings and Trainings
1 committee meeting (October 2019 in Washington, DC), 1 workshop (RIKEN Science Forum workshop, July 2019 in Tokyo, Japan)

Web Tools and Assays
1 web tool (TgX-DDI DNA damage classification tool)
The TgX-DDI is a publicly accessible open source tool that uses genomic data for DNA damage classification (available online via NIEHS at https://manticore.niehs.nih.gov/tgxddi)

Outreach
2 posters, 2 presentations

- Webinar on the "Prediction of Rat Apical Toxicity Points of Departure Using Liver Transcriptome Data" (February 2019)
- Presentation on the "Evaluation of 5-Day *In Vivo* High-Throughput Transcriptomics for Safety Assessment (June 2019)
- Poster on "Preanalytical Impacts of FFPE Specimens on Next Generation Sequencing (NGS) Analysis," presented at the International Society for Biological and Environmental Repositories (November 2019 in Minneapolis, Minnesota)
- Poster on "Improving Formalin-Fixed Paraffin-Embedded (FFPE) Clinical Samples for Variant Calling and Mutant Detection," presented at the International Union of Toxicology (IUTOX) (July 2019 in Honolulu, Hawaii)

Collaborations
1 within HESI (ongoing discussions with the HESI Genetic Toxicology Technical Committee to explore potential collaboration around error-corrected sequencing technology application)

Geographic Representation
Canada, France, Germany, Netherlands, Switzerland, United Kingdom, United States

www.hesiglobal.org

Acknowledgements: Case Study #3

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University of Louisville

Matt Cave

Christina Pinkston

Shesh Rai

Kimberly Head

CDC/ATSDR

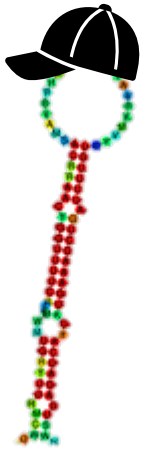
Marian Pavuk

NIEHS

Douglas Bell

Linda Birnbaum

Thank you!

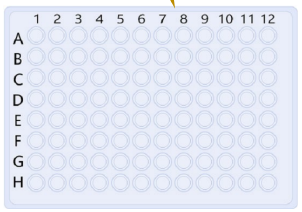


In progress – in vitro application and refinement

- ***Non-destructive measurement of extracellular microRNA to define chemical mode-of-action***

Reference chemicals: Steatotic and other hepatotoxicants

24-48 hr exposure

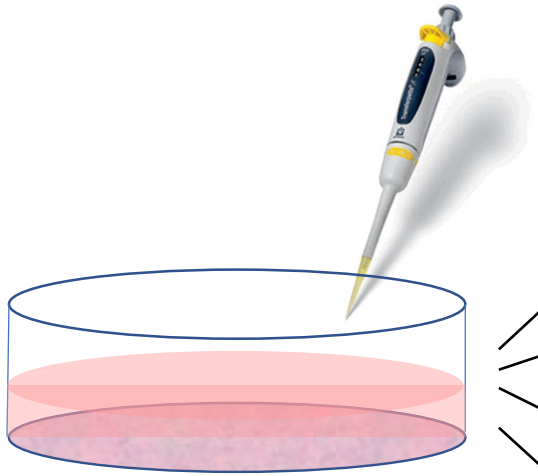


6 hr sample

12 hr sample

24 hr sample

48 hr sample



microRNA released in media (all or exosome)

4 apical KE measurements

AO measurement (Nile Red and CTG)

cellular microRNA and mRNA

- Confirm KE and AO perturbation
- Link to gene expression networks and link cellular microRNA
- Distinguish active and passive microRNA leakage
- Establish extracellular microRNA patterns linked to KE activation and cellular AO
- Link to biosurveillance biomarkers

Exposure | **Sampling** | **Measurements** | **Analyses**