Circulating microRNAs are associated with PCB exposures and liver disease in the Anniston Community Health Survey

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BACKGROUND

- PCB exposures have been associated with liver disease in cohort studies and steatohepatitis with liver necrosis in animal models.
- MicroRNAs (miRs) are non-coding RNAs that are critical regulators of gene expression.
- MiRs are responsive to environmental exposures and alterations may indicate perturbed cellular processes that are linked to later adverse outcomes.
- MiR alterations in cells and tissue can be released extracellularly into serum.
- Therefore, serum miRs may be a useful tool for environmental hepatology cohort studies.

OBJECTIVE

To identify serum-based miRs that are associated with liver disease in a residential cohort exposed to high levels of PCBs.

STUDY DESIGN AND METHODS

- The Anniston Community Health Survey-I (ACHS-I) is a cohort consisting of community participants living near a former PCB production facility in Alabama.
- In ACHS-I, we previously reported a high prevalence of necrotic liver disease associated with exposures to specific PCB congeners, insulin resistance, and pro-inflammatory cytokines - consistent with PCB-related toxicant-associated steatohepatitis (TASH) (PMID:29684222).
- The hepatocyte death biomarker, keratin 18 (K18 M30 and M65, ELISA, DiaPharma); and 35 ortho-substituted PCBs (GC/MS) were measured in de-identified, archived serum (n=738).
- In addition, we measured a panel of 68 hepatotoxicity and fatty liver disease-associated miRs (FirePlex, Abcam).
- Raw mean fluorescent intensities (MFIs) of 30 highly expressed miRs (>LOD in 90+% of the sample) were quantile-normalized and log10-transformed.
- Categorical liver disease variables were created using K18 as follows: no liver disease (K18 M65<300 U/L & M30<200 U/L, n=294); necrotic liver disease (K18 M65>300 U/L & M30<200 U/L, n=359); and other (apoptotic) liver disease (K18 M30>200 U/L, n=85).
- Associations between miRs and log-transformed values of K18 and summed PCBs were determined using generalized, confounder-adjusted linear models.
- Statistical significance was set at a p-value ≤0.05 and/or a false discovery rate (FDR) of ≤0.20.
- Likewise, preliminary data are presented for the ACHS-II re-contact study (n=345). In addition to the 35 ortho-substituted PCBs measured in ACHS-I, ACHS-II measured non-ortho PCBs (n=3), PCDDs (n=7), and PCDFs (n=10) along with additional liver injury biomarkers. Using the WHO TEFs, the toxic equivalencies (TEQs) were determined for PCBs, PCDDs, PCDFs and summed to create the total dioxin TEQ. Adjusted β coefficients were determined for these biomarkers of AhR activation with the miR and other disease biomarkers.

Table 1. Study demographics and liver disease characterization in ACHS-I (n=738)

Characteristic	Liver disease status			P-Value
	None (n = 294)	Necrosis (n = 359)	Other (n = 85)	
Age (years)	54.1±15.7	56.0±16.3[a]	51.5±15.1	**0.04**
BMI (kg/m^2)	31.5±7.8	30.9±7.7	32.1±7.7	0.34
Keratin 18 M65 (U/dL)	233.6±42.6	430.6±122.1[a,b]	792.5±584.9[c]	**<0.001**
Keratin 18 M30 (U/dL)	97.9±22.0	124.0±28.2[a,b]	407.6±324.6[c]	**<0.001**
∑PCBs (whole weight)	6.4±9.1	7.2±14.4	5.4±10.3	0.40
Total lipids (mg/dL)	611.1±131.7	643.6±163.6[b]	656.9±192.4[c]	**0.01**
Sex				**0.03**
Male	72 (24.5)	123[b] (34.3)	26 (30.6)	
Female	222 (75.5)	236 (65.7)	59 (69.4)	
Race/ethnicity				**<0.001**
Non-Hispanic White	117 (39.8)	223[b] (62.1)	53[c] (62.4)	
Nonwhite	177 (60.2)	136 (37.9)	32 (37.7)	

Note: Data are n (%) or mean±SD. Not all percents add to 100% due to rounding. P-value is one-way ANOVA (means) or Pearson chi-square test, across liver disease categories. [a] adj-p<=0.05 in pair-wise comparison of Necrosis vs. Other liver disease categories. [b] adj-p<=0.05 in pair-wise comparison of None vs. Necrosis liver disease categories. [c] adj-p<=0.05 in pair-wise comparison of None vs. Other liver disease categories. Bold font denotes P-value <=0.05. Abbreviation: ACHS-I, Anniston Community Health Survey I; BMI, body mass index; PCBs, polychlorinated biphenyls; SD, standard deviation.

MicroRNA ASSOCIATIONS

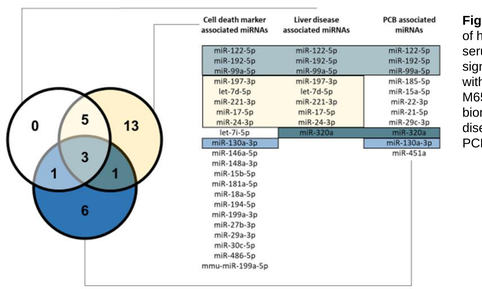


Figure 1. Venn diagram of highly-expressed serum miRs that were significantly associated with the K18 M30 and/or M65 hepatocyte death biomarkers, necrotic liver disease category and/or PCB exposures.

RESULTS

Table 2. Relationships between the differentially regulated, highly-expressed serum miRs and liver disease categories (vs. without liver disease) as well as the serum hepatocyte death biomarker, keratin 18, in a PCB-exposed residential cohort (ACHS-I, 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

Table 3. Associations between other differentially regulated highly-expressed serum miRs which were significantly associated with the serum hepatocyte death biomarker, keratin 18 in ACHS-I.

miRNA	Keratin 18 (n=738)			
	K18 M65		K18 M30	
	β±SE	P-value	β±SE	P-value
miR-181a-5p	**-0.19±0.04**	**<0.0001**	**-0.18±0.04**	**<0.0001**
miR-148a-3p	**0.15±0.03**	**<0.0001**	**0.13±0.03**	**0.0002**
miR-30c-5p	**-0.14±0.03**	**<0.0001**	**-0.15±0.03**	**<0.0001**
miR-18a-5p	**-0.19±0.05**	**<0.0001**	**-0.17±0.05**	**0.0002**
mmu-miR-199a-5p	**-0.20±0.05**	**<0.0001**	**-0.21±0.05**	**<0.0001**
miR-27b-3p	**-0.17±0.04**	**0.0001**	**-0.09±0.04**	**0.04**
miR-199a-3p	**-0.13±0.04**	**0.0008**	**-0.11±0.04**	**0.004**
miR-15b-5p	**-0.06±0.02**	**0.001**	**-0.07±0.02**	**0.0001**
miR-29a-3p	**0.10±0.03**	**0.002**	**0.10±0.03**	**0.001**
miR-194-5p	**0.12±0.05**	**0.006**	**0.11±0.04**	**0.01**
miR-130a-3p	**-0.07±0.03**	**0.008**	**-0.08±0.03**	**0.002**
let-7i-5p	**-0.07±0.03**	**0.02**	**-0.06±0.03**	**0.04**
miR-486-5p	**-0.07±0.03**	**0.03**	**-0.06±0.03**	**0.03**
miR-146a-5p	**-0.07±0.03**	**0.04**	-0.05±0.03	0.10

Table 4. Associations between highly-expressed serum miRs with whole weight polychlorinated biphenyls (PCBs) as thirty-five summed ortho-substituted congeners and as selected individual estrogenic PCB congeners in ACHS-I (n-738)

miRNA	∑PCBs		Selected estrogenic PCB congeners									
			PCB28		PCB44		PCB49		PCB52		PCB101		PCB110	
	β±SE	P-value	β±SE	P-value	β±SE	P-value	β±SE	P-value	β±SE	P-value	β±SE	P-value	β±SE	P-value
Up-regulated miRNA														
miR-122-5p	-0.04±0.05	0.47	**0.12±0.05**	**0.01**	**0.36±0.1**	**0.0002**	**0.29±0.08**	**0.001**	**0.21±0.06**	**0.0002**	**0.12±0.05**	**0.01**	**0.11±0.06**	**0.04**
miR-192-5p	-0.02±0.03	0.48	0.06±0.03	0.07	0.11±0.06	0.07	0.06±0.05	0.28	0.05±0.04	0.20	**0.09±0.03**	**0.003**	0.06±0.03	0.07
miR-320a	0.01±0.01	0.61	-0.01±0.01	0.66	0.01±0.03	0.84	0.00±0.03	0.99	0.02±0.02	0.36	0.02±0.01	0.20	0.00±0.02	0.79
miR-99a-5p	0.01±0.03	0.73	**0.06±0.03**	**0.02**	0.08±0.05	0.13	0.08±0.05	0.09	**0.07±0.03**	**0.04**	0.03±0.03	0.31	0.03±0.03	0.28
Down-regulated miRNA														
miR-24-3p	-0.02±0.02	0.34	-0.01±0.02	0.45	-0.05±0.03	0.16	-0.02±0.03	0.58	-0.01±0.02	0.69	0.00±0.02	0.93	-0.02±0.02	0.24
miR-197-3p	0.01±0.03	0.69	-0.02±0.03	0.38	-0.02±0.05	0.64	0.01±0.04	0.86	0.01±0.03	0.73	0.00±0.02	0.95	0.01±0.03	0.86
let-7d-5p	-0.01±0.02	0.71	0.00±0.02	>0.99	-0.01±0.04	0.86	-0.03±0.03	0.36	-0.01±0.02	0.59	0.00±0.02	>0.99	0.02±0.02	0.45
miR-221-3p	-0.01±0.02	0.51	-0.03±0.02	0.12	-0.02±0.04	0.63	0.04±0.03	0.21	-0.01±0.02	0.68	-0.03±0.02	0.10	-0.01±0.02	0.63
miR-17-5p	-0.01±0.01	0.33	0.00±0.01	0.86	0.00±0.03	0.85	0.00±0.02	0.93	-0.01±0.02	0.48	0.00±0.01	0.82	-0.01±0.01	0.67
Keratin 18 differentially regulated miRNAs														
miR-130a-3p	-0.03±0.02	0.09	0.00±0.02	0.79	**-0.07±0.03**	**0.03**	-0.03±0.03	0.36	-0.03±0.02	0.18	-0.03±0.02	0.10	-0.01±0.02	0.56
Other miRNAs of Interest														
miR-29c-3p	0.00±0.03	0.91	-0.02±0.03	0.54	**-0.12±0.05**	**0.01**	**-0.10±0.04**	**0.02**	**-0.07±0.03**	**0.03**	-0.03±0.02	0.29	-0.03±0.03	0.31
miR-185-5p	-0.01±0.02	0.59	-0.01±0.02	0.51	-0.03±0.04	0.41	**-0.08±0.03**	**0.01**	-0.04±0.02	0.08	-0.02±0.02	0.33	-0.03±0.02	0.15

Abbreviations: ACHS-I, Anniston Community Health Survey I; let, lethal; miRNA/miR, microRNA; PCBs, polychlorinated biphenyls; SE, standard error. **Note:** PCB99 and PCB128 also have estrogenic activity but were not associated with the miRs provided in Table 4. However, PCB99 was significantly associated miR-21-5p (β=0.04±0.02, p=0.046) and miR-451a (β=-0.03±0.01, p=0.02). See Supplemental Table 5 for associations between miRs and non-estrogenic PCB congeners.

Table 5. Enriched toxicity (Panel Bfunctions elucidated by the differentially regulated miRs associated with the necrotic liver disease category (Panel A) or PCB exposures) in ACHS-I. Range for P-value is shown if more than one pathway is enriched for a given category.

(A) Necrotic liver disease-associated miRNAs

Enriched tissue specific toxicity	p-value	Associated miRNAs
Liver Tissue		
Hepatocellular carcinoma	3.02E-06 - 1.1E-02	let-7d-5p, miR-99a-5p, miR-122-5p, miR-17-5p, miR-192-5p, miR-221-3p
Liver Hyperplasia/Hyperproliferation	3.02E-06 - 1.1E-02	let-7d-5p, miR-99a-5p, miR-122-5p, miR-17-5p, miR-192-5p, miR-221-3p
Liver Inflammation/Hepatitis	2.75E-04 - 2.75E-04	miR-99a-5p, miR-221-3p
Liver Cirrhosis	4.74E-03 - 4.74E-03	miR-99a-5p, miR-221-3p

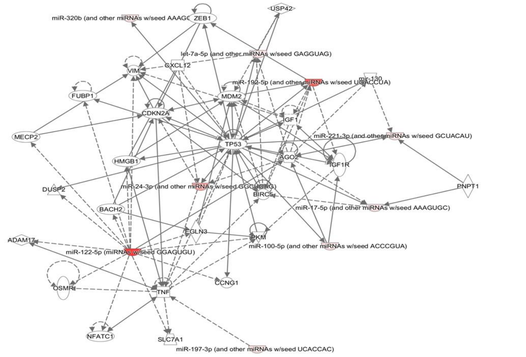
(B) PCB-associated miRNAs

Enriched tissue specific toxicity	p-value	Associated miRNAs
Liver Tissue		
Hepatocellular carcinoma	2.03E-08 - 1.35E-02	miR-99a-5p, miR-122-5p, miR-130a-3p, miR-15a-5p, miR-192-5p, miR-21-5p, miR-22-3p, miR-29c-3p
Liver Hyperplasia/Hyperproliferation	2.03E-08 - 1.35E-02	miR-99a-5p, miR-122-5p, miR-130a-3p, miR-15a-5p, miR-192-5p, miR-21-5p, miR-22-3p, miR-29c-3p
Liver Inflammation/Hepatitis	3.41E-06 - 3.41E-06	miR-99a-5p, miR-130a-3p, miR-15a-5p
Liver Cirrhosis	2.51E-04 - 2.51E-04	miR-99a-5p, miR-130a-3p, miR-15a-5p

Table 6. Associations between miRs and Biomarkers of Dioxin-like Molecule Exposures and Other Disease Biomarkers in ACHS-II (n=345)

microRNA	Toxic Equivalency Biomarkers	Selected ΣPCB Biomarkers	Liver Injury Biomarkers	Intermediary Metabolism Biomarkers	Adipocytokine and Intestinal Permeability Biomarkers
miR-29a-3p	Total Dioxin TEQ (β=-0.16±0.05) MO-PCB TEQ (β=-0.08±0.03) NO-PCB TEQ (β=-0.08±0.03)	ΣPCB (β=-0.07±0.04) ΣDL (β=-0.07±0.03) ΣNDL(β=-0.07±0.04)	K18 M30 (β=0.09±0.05)	Total Protein (β=0.95±0.35) Albumin (β=0.62±0.30) Total Cholesterol (β=-0.67±0.25) Triglycerides (β=0.18±0.08) HOMA-IR (β=0.07±0.03)	Adiponectin (β=-0.11±0.04) Endotoxin (β=0.11±0.03)
miR-185-5p	Total Dioxin TEQ (β=-0.07±0.04)		Direct Bill (β=0.45±0.09)		TNFα (β=-0.06±0.03) PAI-1 (β=-0.14±0.06)
miR-451a	PCDD TEQ (β=-0.11±0.05) PCDF TEQ (β=-0.11±0.05)		K18 M30 (β=-0.09±0.04) Alk Phos (β=-0.2±0.08)	HOMA-B (β=0.08±0.02)	Resistin (β=-0.10±0.05)
miR-320a	PCDD TEQ (β=0.08±0.04) PCDF TEQ (β=0.08±0.04)		K18 M30 (β=0.06±0.03) Alk Phos (β=0.18±0.06)	Total Cholesterol (β=-0.43±0.16) Triglycerides (β=0.16±0.06) HOMA-B (β=-0.04±0.02)	Resistin (β=0.12±0.03) Adiponectin (β=0.06±0.03) Endotoxin (β=-0.04±0.02)
miR-27b-3p	PCDD TEQ (β=-0.18±0.08)				
let7d-5p	PCDF TEQ (β=-0.13±0.06)		K18 M30 (β=-0.14±0.05)	Total Protein (β=-0.82±0.38)	Resistin (β=0.17±0.06)
miR-194-5p	PCDF TEQ (β=0.19±0.08)		Direct Bill (β=0.46±0.09) AST (β=0.29±0.11)		Adiponectin (β=0.14±0.06) Endotoxin (β=0.13±0.04)
miR-197-3p	PCDF TEQ (β=0.15±0.07)				
miR-146b-5p	PCDF TEQ (β=-0.21±0.07)				Endotoxin (β=-0.10±0.04)

Figure 2. Ingenuity Pathway Analysis (IPA) network of the highly-expressed serum miRs associated with categorical liver disease in ACHS-I. Red scale (from light to deep red) indicates decreasing p-value associated with liver disease. Arrows between nodes represent direct (solid lines) and indirect (dashed lines) interactions.



CONCLUSIONS

- The prevalence of necrotic liver disease was 48.6% in ACHS-I and 45.8% in ACHS-II.
- In ACHS-I, necrotic liver disease, serum K18 and PCB congeners were associated with 9, 22 and 11 circulating miRs previously associated with liver toxicity, respectively.
- In ACHS-I, three miRS were positively associated with categorical liver disease, continuous K18 and PCB exposures. These miRs included: miR-122-5p, miR-192-5p, and miR-99a-5p. The associations were most significant for the liver-specific miR-122-5p. The associations between miR-122-5p and the necrotic liver disease category and K18 biomarkers was confirmed in ACHS-II (data not shown).
- In ACHS-I, several liver toxicity miRS were associated with estrogenic PCB congeners.
- The enriched hepatotoxicity functions by IPA for the miRNAs associated with the necrotic liver disease category, continuous K18 (not shown) and the PCB exposures were identical. These functions included: liver inflammation/hepatitis, cirrhosis, hyperplasia/hyperproliferation and hepatocellular carcinoma.
- P53 was central to the networks enriched by the miRs associated with the necrotic liver disease category (not shown), continuous K18 (not shown), and PCB exposures.
- In ACHS-II, total dioxin TEQ was associated with two miRs (miR-29a-3p and miR-185-5p). PCB TEQ was associated only with miR-29a-3p. PCDD TEQ was associated with 3 miRS (miR-451a, miR-320a and miR-27b-3p). PCDF TEQ was associated with six miRs (miR-451a, miR-320a, let7d-5p, miR-194-5p, miR-197-3p, and miR146b-5p.
- Toxic equivalency-associated miRs were also associated with additional biomarkers of liver injury, intermediary metabolism, intestinal permeability and adipocytokines.
- The results broadly support the K18-based liver disease categorization procedures (in PMID29684222).
- The potential role of miRs associated with PCB and dioxin exposure in liver cell death, inflammation, fibrosis, HCC, endocrine disruption and systemic inflammation warrant further investigation.
- Likewise, estrogenic PCBs, P53 and the AhR require further investigation in environmental liver diseases.
- Reverse causality has not been excluded, but serum miRs appear to be a useful tool for environmental hepatology cohort studies.

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