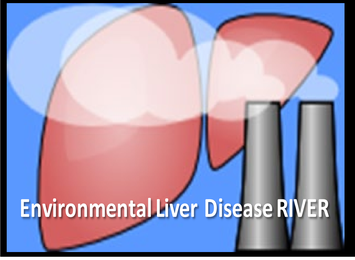


Serum microRNA Profiling Yields Mechanistic Insight into a Residential Cohort with Environmental Liver Disease

Cave MC[1], Pinkston CM[1], Rai SN[1], Carswell G[2], Nelson GM[2], Head KZ[1], Wahlang B[1], Saad YM[1], Pavuk M[3], Chorley BN[2]

[1]University of Louisville, Louisville, KY 40245; [2]US Environmental Protection Agency, Research Triangle Park, NC 27711;
[3]Agency for Toxic Substances and Disease Registry, Centers for Disease Control and Prevention. Atlanta, GA 30341, USA

ABSTRACT

Background: Polychlorinated biphenyls (PCBs) are industrial pollutants previously associated with steatohepatitis. 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). MicroRNAs (miRs) are non-coding RNAs that maintain cellular homeostasis. A pilot study demonstrated the potential utility of serum miR profiling in ACHS-I (n=152, DDW 2018, 7110A). Based on those preliminary data, hepatotoxicity miRs were measured in all available ACHS-I participants**. Methods:** IRB-approval was obtained. A panel of 68 targeted hepatotoxicity miRs (FirePlex, Abcam); 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). Raw mean fluorescent intensities (MFIs) of 30 highly expressed miRs (>LOD in 90+% of the sample) were quantile-normalized and log10-transformed. Values <LOD were assigned LOD/√2. 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 apoptotic liver disease (K18 M30>200 U/L, n=85). For miRs, fold-change was calculated by taking the exponential power of the ratio of the back-transformed MFI values for the two groups with liver disease relative to the no liver disease control group. Associations between miRs and log-transformed values of K18 and summed PCBs were determined using generalized, confounder-adjusted linear models. Data analysis was performed using R (R Core Team), SAS v9.4 (SAS Institute, Cary, NC. USA), and Ingenuity Pathway Analysis (IPA, Qiagen, Hilden, Germany). Statistical significance was set at a p-value ≤0.05 and/or a false discovery rate (FDR) of ≤0.20. **Results:** Demographic information was previously published (PMID:29684222). Necrotic liver disease was associated with 9 miRs including: miR122-5p (1.46-fold, FDR<.0001); miR192-5p (1.20-fold, FDR=0.0003); miR320a (1.05-fold, FDR=0.15); miR99a (1.09-fold, FDR=0.17); miR17-5p (0.96-fold, FDR=0.14); miR221-3p (0.94-fold, FDR=0.14); let7d-5p (0.94-fold, FDR=0.12); miR24-3p (0.91-fold, FDR=0.02); and miR197-3p (0.91-fold, FDR=0.09). Apoptotic liver disease was associated with 7 miRs including: miR122-5p (2.91-fold, FDR<.0001); miR192-5p (1.64-fold, FDR<0.0001); miR99a-5p (1.38-fold, FDR<0.0001); miR221-3p (0.84-fold, FDR=0.003); miR1973p (0.88-fold, FDR=0.14) and let7d-5p (0.82-fold, FDR=0.0003). None of these miRs were associated with ΣPCBs. 22 miRs were associated with continuous k18 values, and 11 miRs were associated with 24 PCB congener exposures. IPA demonstrated enrichment in liver toxicity functions (inflammation/hepatitis, hyperplasia/hyperproliferation, cirrhosis and hepatocellular carcinoma) for the miRNAs associated with either liver disease category, K18 or PCB exposures. Likewise, P53 was centrally located in the network analyses for these miRNAs.
Discussion: The miR results broadly support the liver disease categorization procedures using K18, which demonstrated an exceptionally high liver disease prevalence in ACHS-1 (60.2%, PMID:29684222), but lacked histologic confirmation. Necrotic and apoptotic liver diseases were associated with slightly different serum miR profiles. The potential impact of environmental PCB exposures on hepatic inflammation, cell death, fibrosis, P53 and hepatocellular carcinoma warrants further investigation. This does not necessarily reflect EPA or ATSDR policy.

ACHS-I DEMOGRAPHICS

Table 1. Demographic variables and liver disease categorization 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)	

Abbreviations: ACHS-I, Anniston Community Health Survey I; BMI, body mass index; PCBs, polychlorinated biphenyls; SD, standard deviation.
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.

MicroRNA Associations

Table 2. Relationships between the differentially regulated highly-expressed serum miRNAs and liver disease categories (*vs.* without liver disease) as well as serum keratin 18 in a residential cohort with elevated exposure to polychlorinated biphenyls (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 miRNAs which were significantly associated with serum keratin 18 but not categorical liver disease in ACHS-I (n=738).

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 miRNAs 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.

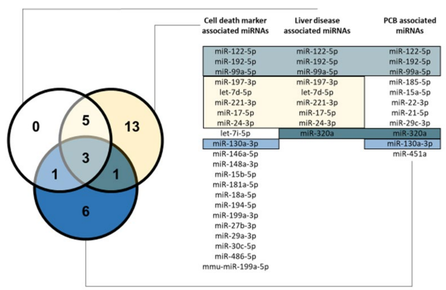


Table 5. Associations between non-estrogenic polychlorinated biphenyls (whole weight) and differentially regulated and highly-expressed microRNAs in ACHS-I (n=738)

Non-Estrogenic PCB Congeners	Differentially Regulated microRNAs											
	miR-15a-5p		miR-22-3p		miR-130a-3p		miR-185-5p		miR-320a		miR-451a	
	β±SE	P-value	β±SE	P-value	β±SE	P-value	β±SE	P-value	β±SE	P-value	β±SE	P-value
PCB66			0.04±0.01	0.01							-0.03±0.01	0.04
PCB87					-0.03±0.01	0.048						
PCB105											-0.02±0.01	0.05
PCB118											-0.03±0.01	0.02
PCB151			0.04±0.01	0.002			-0.04±0.02	0.02	0.03±0.01	0.03	-0.03±0.01	0.01
PCB153											-0.03±0.01	0.0498
PCB156	0.04±0.02	0.03										
PCB157	0.03±0.02	0.03										
PCB167											-0.03±0.01	0.03
PCB170	0.03±0.02	0.04										
PCB177	0.03±0.01	0.05										
PCB194	0.03±0.01	0.03			-0.03±0.01	0.02						
PCB195	0.03±0.01	0.03										
PCB196					-0.03±0.02	0.04						
PCB199	0.04±0.01	0.01			-0.03±0.01	0.04						
PCB206	0.03±0.01	0.01			-0.03±0.01	0.03						
PCB209	0.04±0.01	0.01			-0.03±0.01	0.01						

Abbreviations: ACHS-I, Anniston Community Health Survey I; miR, microRNA; PCB, polychlorinated biphenyl; SE, standard error.

Table 6. Enriched toxicity functions elucidated by the differentially regulated miRNAs associated with the necrotic liver disease category (Panel A); the K18 M30 and/or M65 hepatocyte death biomarkers (Panel B); or PCB exposures (Panel C).

(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) K18-associated miRNAs

Enriched tissue specific toxicity	p-value	Associated miRNAs
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

(C) PCB congener-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

Figure 2. IPA network analysis of the highly-expressed serum microRNAs associated with categorical liver disease in ACHS-I.

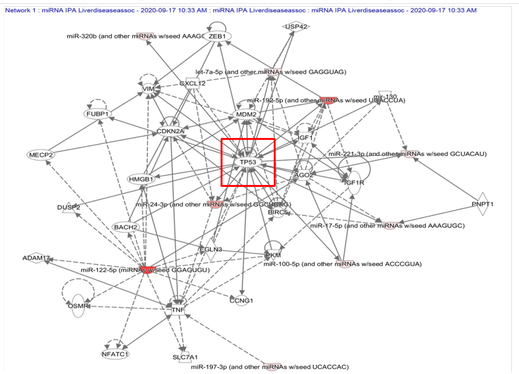
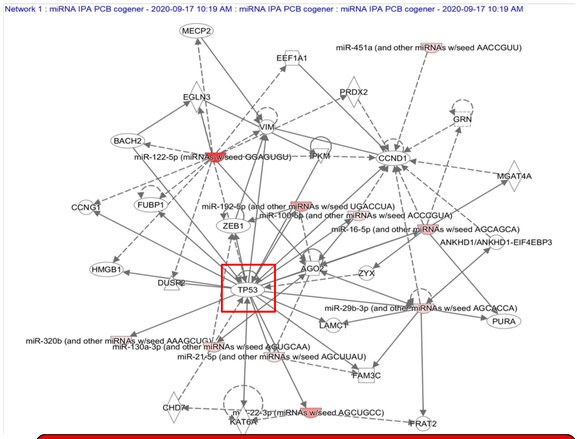


Figure 3. IPA network analysis of the highly-expressed serum microRNAs associated with serum PCB congeners in ACHS-I.



Conclusions

- Necrotic liver disease, continuous serum K18 and PCB congeners were associated with 9, 22 and 11 circulating microRNAs, respectively.
- Three miRS were positively associated with categorical liver disease, continuous K18 and PCB exposures. These miRs included: miR122-5p, miR192-5p, and miR99a-5p.
- The enriched hepatotoxicity functions by IPA for the miRNAs associated with the necrotic liver disease category, continuous K18 and the PCB exposures were identical. These functions included: liver inflammation/hepatitis, cirrhosis, hyperplasia/hypoproliferation and hepatocellular carcinoma.
- P53 was central to the networks enriched by the miRs associated with the necrotic liver disease category, continuous K 18 (not shown), and PCB exposures.
- The results broadly support the K18-based liver disease categorization procedures which demonstrated a 60.2% liver disease prevalence in ACHS-I (PMID29684222).
- The potential role of PCB-regulated microRNAs in liver cell death, inflammation, fibrosis and HCC warrant further investigation.
- Likewise, estrogenic PCBs and P53 require further investigation in environmental liver diseases.
- Reverse causality has not been excluded.
- This does not necessarily reflect EPA or ATSDR policy.

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