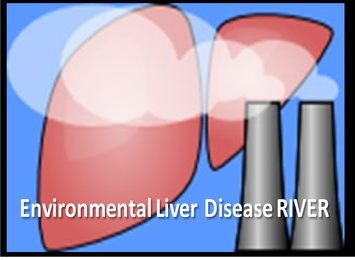

MicroRNA Liver Toxicity Biomarkers Associated with Dioxin-Like Molecule Exposures in ACHS-II

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BACKGROUND

- Associations between environmental polychlorinated biphenyl (PCB) exposures and liver/metabolic diseases and epigenetic marks, including circulating microRNAs (miRs), have been reported in the Anniston Community Health Survey (ACHS-I & -II) (PMID:29684222, 31607210, 34989596).
- MiRs are non-coding RNAs that are critical regulators of gene expression that are responsive to environmental exposures. Alterations in miRs may indicate perturbed cellular processes that are linked to later adverse outcomes, including liver disease.
- Exposures to dioxin-like pollutants (e.g., polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) have reproducibly been associated with metabolic disruption and NAFLD in animal models (PMID: 31134516).

OBJECTIVE

To determine cross-sectional relationships between circulating liver toxicity biomarkers, including miRs, and dioxin-like molecules (e.g., non-ortho PCBs, PCDDs and PCDFs) in the follow-up ACHS-II cohort.

STUDY DESIGN AND METHODS

- The ACHS is a longitudinal cohort consisting of adult community participants living near a former PCB production facility in Anniston, Alabama.
- The design of the ACHS-II follow-up study was previously reported (PMID:25982988). A sample size of 345 subjects was utilized in this analysis of de-identified previously collected samples, which was IRB approved.
- A total of twenty dioxin-like molecules (non-ortho PCBs (3), PCDDs (7), and PCDFs (10)) were measured by gas chromatography isotope dilution high-resolution mass spectrometry (GC/ID-HRMS) in serum as previously reported (PMID:29763869). Dioxin toxic equivalency (TEQ), a biomarker of aryl hydrocarbon receptor by dioxin-like molecules, was determined using World Health Organization toxic equivalency factors (PMID:16829543) for each of the three pollutant categories as well as the summed total (total dioxin TEQ). The total dioxin TEQ was previously reported to be significantly higher in ACHS-II than NHANES (PMID:29763869). 35 ortho-substituted PCBs were likewise measured and the mono-ortho PCB (MO-PCB) TEQ was similarly determined and the sum of these 35 congeners reported as well as ∑PCBs.
- Liver injury/disease biomarkers were measured in serum including: (*i*) liver enzymes (clinical chemistry analyzer); (*ii*) keratin 18 M30 & M65 (ELISA, DiaPharma); (*iii*) a panel of 68 hepatotoxicity miRs in a customized panel (FirePlex, Abcam); and (*iv*) an adipocytokine multiplex panel (Milliplex, Millipore).
- Raw mean fluorescent intensities (MFIs) of 35 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); necrotic liver disease (K18 M65>300 U/L & M30<200 U/L); and other (apoptotic) liver disease (K18 M30>200 U/L).
- Associations between log-transformed exposure and disease biomarkers were determined using generalized, confounder-adjusted linear models using SAS v9.4 and R and presented as beta coefficients.
- Ingenuity Pathway Analysis (IPA) of associated miRs was performed.
- Statistical significance was set at a p-value ≤0.05 and/or a false discovery rate (FDR) of ≤0.10 for the primary outcome (associations between miRs and liver disease categories) and p<0.05 for secondary outcomes.
- More details regarding these methodologies can be found in our previous publications on liver disease, cytokines and miRs in ACHS-I (PMID: 29684222, 34989596).

SUBJECT CHARACTERISTICS

Table 1. Demographic characteristics by K18-categorized liver disease status (N=345).

Characteristic	Liver disease status[a, b] None (N = 131)		Necrotic (N = 158)		Apoptotic (N = 56)		P-value[c]
Age (years)	63.8	12.7	63.4	13.2	59.8	12.6	0.13
BMI (kg/m²)	32.0	9.5	31.2	7.2	32.2	7.2	0.59
Keratin 18 M65 (U/dL)[d]	231.8	46.0	436.7	142.9	624.8	368.1	**<.0001**
Keratin 18 M30 (U/dL)[d]	84.1	26.5	111.6	36.3	380.1	269.8	**<.0001**
∑PCBs (whole weight)	6.0	6.4	5.4	7.2	5.6	9.8	0.17
Total TEQ (whole weight)	164.8	129.9	144.1	139.9	142.5	174.6	**0.04**
Total lipids (mg/dL)	611.1	143.7	632.5	162.1	622.1	154.8	0.47
Sex							0.07
Male	26	(19.9)	49	(31.0)	18	(32.1)	
Female	105	(80.2)	109	(69.0)	38	(67.9)	
Race/ethnicity							**0.006**
Non-Hispanic White	50	(38.2)	90	(57.0)	28	(50.0)	
Nonwhite	81	(61.8)	68	(43.0)	28	(50.0)	

Abbreviations: dL, deciliter; K18, Keratin 18; kg, kilograms; m, meters; mg, milligrams; PCB, polychlorinated biphenyl; SD, standard deviation; TEQ, toxic equivalency; U, units.
[a] Liver disease categories are defined as: none (K18 M30 < 200 and K18 M65 < 300), necrotic (K18 M30 < 200 and K18 M65 ≥ 300), and other abnormal K18 (K18 M30 ≥ 200).
[b] Units are in Mean±SD or N (%) for continuous or categorical values, respectively.
[c] P-values for continuous characteristics are based on a one-way ANOVA using $\log_{10}$-transformed values where ∑PCBs and total TEQ are adjusted for $\log_{10}$-transformed total lipids.
[d] Each pairwise comparison differed significantly: p<0.0001.

RESULTS

A. Liver Disease Prevalence: Consistent with ACHS-I (PMID:29684222), there was a high prevalence of K18-categoraized liver disease (62.0%), with 61.2% of the total being necrotic and 38.8% being apoptotic (**Table 1**). The K18-categorized liver disease groups were associated with increased liver enzymes (**Table 2**).

Table 2. Mean Liver Enzymes Stratified by Liver Disease Status, ACHS II (n=345)

	None		Necrotic		Apoptotic			Total	
	Mean	SD	Mean	SD	Mean	SD	P-Value	Mean	SD
AST (U/L)	25.9	8.0	29.9	14.5	42.1	27.2	**<.0001**	30.4	16.4
ALT (U/L)	22.8	8.6	26.5	9.8	38.1	19.2	**<.0001**	26.9	12.6
Alkaline phosphatase (U/L)	85.4	22.8	91.1	38.7	105.7	57.9	**0.004**	91.3	38.3
Albumin (g/dL)	4.2	0.4	4.1	0.4	4.2	0.5	0.78	4.2	0.4
Total Protein	7.3	0.6	7.2	0.6	7.4	0.7	0.053	7.3	0.6
Total bilirubin (mg/dL)	0.4	0.2	0.5	0.5	0.5	0.2	**0.03**	0.4	0.4
Direct bilirubin (mg/dL)	0.2	0.1	0.2	0.2	0.2	0.1	0.47	0.2	0.2

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; dL, deciliters; g, grams; mg, milligrams; L, liters; SD, standard deviation; U, units.

B. Relationships Between Liver Injury/Disease Biomarkers: 35 of 68 circulating miRs were highly-expressed. Associations between these MiRs, liver enzymes, and K18 were determined. Twenty-nine miRs were significantly associated with at least 1 other liver injury biomarker (not shown). MiR-122-5P was associated with M30, M65, AST, ALT, and alkaline phosphatase (all p<0.01) with a trend towards total protein (p=0.06). MiR-192-5P was associated with the same variables (all p<0.03), except total protein, as well as direct bilirubin (p=0.003).

Using complete linkage analysis, miRs, K18 and liver enzyme disease biomarkers clustered into four groups (G): G1 (n=9 miRs), G2 (n=4), G3 (n=11), G4 (n=11). G2 miRs were associated with the liver enzyme, K18 and the synthetic function biomarkers (**Figure 1**). Using Ingenuity Pathway Analysis, miRs in the four groups were linked to various liver disease processes including: hepatic steatosis (G2), hepatitis (G4), cirrhosis (G4), liver hyperplasia (G1-G2), and hepatocellular carcinoma (G1-G3) (**Figure 2**).

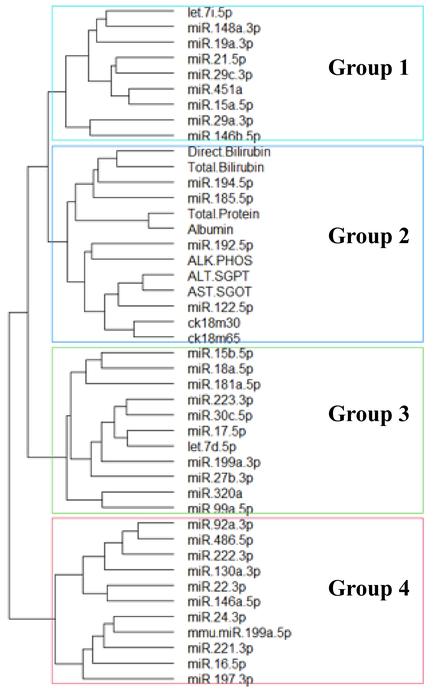
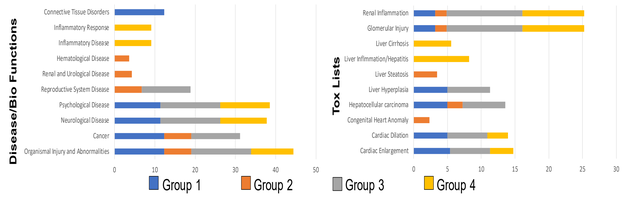


Figure 1. Using complete linkage analysis, serum miRs and liver tests clustered into four groups (G): G1 (n=9 miRs), G2 (n=4), G3 (n=11), G4 (n=11). G2 miRs were associated with the liver enzyme and K18 biomarkers.

Figure 2. Ingenuity pathway analysis (IPA) of miRs from the four liver biomarker groups determined by complete linkage analysis (n=345). Top 5 pathways for Diseases and Bio Functions and top 2 pathways for liver, renal, and cardiac tox lists are shown. X-axis represents –log(p-value) of pathway enrichment.



C. Relationships miRs and Liver Disease Categories: Associations between differentially regulated highly-expressed serum miRs which were significantly associated with the liver disease categories in ACHS-II were determined (**Table 3**). Necrotic liver disease was significantly associated with increased miR-122-5p (group 2, p=0.01 and FDR=0.09). Apoptotic liver disease was associated with significantly increased miR-122-5p, miR-192-5p (group 2), miR-99a-5p (group 3), and miR-22-3p (group 4); and significantly decreased miR-18a-5p (group 3), miR199a-3p (group; 3), and let7d-5p (group 3) (all with p<=0.01 and FDR<-0.09). Some associated present ACHS-I were not present in ACHS-II (e.g., miR-320a, miR-221-3p, miR24-3p, miR-17-5p, miR-197-3p. These data confirm the key results from ACHS-I (e.g., miRs-122, -192, and -99).

Table 3. Differentially regulated serum miRs and liver disease categories (vs. without liver disease) in a PCB-exposed residential cohort study (ACHS-I, n-738; ACHS-II, n=345).

miR	Differentially regulated miRs in necrotic liver disease (N=158) FC	SE	FDR	P_{raw}	Differentially regulated miRs in apoptotic liver disease (N=56) FC	SE	FDR	P_{raw}
Up-regulated miRs								
miR-122-5p	**1.36**	**0.16**	**0.09**	**0.01**	**2.69**	**0.42**	**<.0001**	**<.0001**
miR-192-5p	1.08	0.11	0.80	0.41	**1.61**	**0.21**	**0.01**	**0.0002**
miR-99a-5p	1.05	0.11	0.80	0.64	**1.43**	**0.19**	**0.09**	**0.01**
miR-22-3p[a]	1.03	0.06	0.80	0.59	**1.24**	**0.10**	**0.09**	**0.01**
Down-regulated miRs								
miR-18a-5p[a]	0.97	0.09	0.90	0.77	**0.74**	**0.08**	**0.09**	**0.01**
miR-199a-3p[a]	0.96	0.06	0.80	0.54	**0.78**	**0.07**	**0.09**	**0.01**
let7d-5p[a]	1.03	0.07	0.80	0.60	**0.80**	**0.07**	**0.01**	**0.01**
Differentially regulated miRs in ACHS-I[b]								
miR-320a	0.97	0.04	0.80	0.45	1.08	0.06	0.54	0.17
miR-221-3p	0.97	0.06	0.80	0.60	0.88	0.07	0.54	0.11
miR-24-3p	0.96	0.05	0.80	0.49	0.89	0.06	0.54	0.10
miR-17-5p	0.98	0.06	0.90	0.77	0.93	0.08	0.80	0.38
miR-197-3p	0.98	0.08	0.95	0.84	0.95	0.10	0.80	0.65

Note: Statistical significance was set at FDR <0.20 for the primary endpoint (categorical liver disease associations) and P-value <0.05 for all secondary endpoints. Bold font denotes statistically significant results. **Abbreviations:** ACHS-I, Anniston Community Health Survey I; ACHS-I, ACHS Survey II; FC, fold change (vs. the no liver disease category); FDR, false discovery rate; K18, Keratin 18; let, lethal; miR, microRNA; PCB, polychlorinated biphenyls; SE, standard error. **Model:** Adjusted for age, BMI, race sex, miR plate. [a] Significant in ACHS-II only. [b] Significant in ACHS-I only

C. Relationships between dioxin exposures and liver toxicity biomarkers.

- Total dioxin TEQ was significantly associated with liver toxicity biomarkers from group 1 (miR-29a-3p) and group 2 (miR-185-5p, albumin and total protein) (**Table 4**). All of these relationships were inverse.
- PCDD TEQ was significantly associated with liver toxicity biomarkers from group 2 (albumin) and group 3 (miR-27b-3p and miR-320a) (**Table 4**).
- PCDF TEQ was significantly associated with a total seven liver toxicity biomarkers coming from all four groups and including albumin and miR-320a (**Table 4**).
- Non-ortho PCB TEQ was inversely associated with miR-29a-3p and albumin. Other PCB biomarkers were also inversely associated with miR-29a-3p, although ΣPCBs was not associated with any liver toxicity biomarker (**Table 4**).
- Relationships between key miRs associated with exposures to dioxin-like molecules with biomarkers of liver toxicity, intermediary metabolism and adipocytokines are provided in **Table 5**.

Table 4. Selected Significant Associations Between Dioxin Exposure and Liver Toxicity Biomarkers

Exposure Biomarker	Liver Toxicity Biomarker (Group 1)	Liver Toxicity Biomarker (Group 2)	Liver Toxicity Biomarker (Group 3)	Liver Toxicity Biomarker (Group 4)
Total Dioxin TEQ	**miR-29a-3p** (β= -0.16±0.05, p=0.0004)	**Albumin** (β= -0.02±0.01, p=0.03) **Total Protein** (β= -0.02±0.01, p=0.03) **miR-185-5p** (β= -0.07±0.04, p=0.049)	-	-
PCDD TEQ	-	**Albumin** (β= -0.02 ±0.01, p=0.03)	**miR-27b-3p** (β= -0.18±0.09, p=0.04) **miR-320a** (β= 0.08±0.04, p=0.04)	-
PCDF TEQ	**miR-146b-5p** (β= -0.21±0.07, p=0.003) **miR-451a** (β= -0.11±0.05, p=0.02)	**Albumin** (β= -0.02±0.01, p=0.04) **miR-194-5p** (β= 0.19±0.08, p=0.02)	**miR-320a** (β= 0.08±0.04, p=0.02) **let-7d-5p** (β= -0.13±0.06, p=0.03)	**miR-197-3p** (β=0.15±0.07, p=0.04)
Non-ortho PCB TEQ	**miR-29a-3p** (β= -0.08±0.03, p=0.01)	**Albumin** (β= -0.01±0.01, p=0.04)	-	-
Mono-ortho PCB TEQ	**miR-29a-3p** (β= -0.08±0.03, p=0.02)	-	-	-
∑Non-dioxin like PCBs	**miR-29a-3p** (β= -0.07±0.04, p=0.04)	-	-	-
ΣPCBs	-	-	-	-

Table 5. Selected Significant Associations Between TEQ-Associated miRs and Liver Toxicity Biomarkers or Metabolic/Cytokine Biomarkers

TEQ-Associated miRs	Liver Toxicity Biomarkers	Metabolic & Cytokine Biomarkers
miR-29a-3p	**K18 M30** (β= 0.09±0.05, p=0.04) **Albumin** (β= 0.62±0.31, p=0.04) **Total Protein** (β= 0.96±0.35, p=0.01)	**HDL** (β= -0.30±0.10, p=0.01) **VLDL** (β= 0.13±0.06, p=0.02) **HOMA-IR** (β= 0.07±0.03, p=0.04) **CCXL11** (β= 0.07±0.03, p=0.03) **Endotoxin** (β= 0.11±0.03, p=0.0003) **Adiponectin** (β= -0.11±0.04, p=0.01)
miR-185-5p	**Direct Bilirubin** (β= 0.18±0.05, p=0.001)	**LDL** (β= 0.14±0.06, p=0.02) **PAI-1** (β= 0.19±0.06, p=0.03) **TNFα** (β= -0.10±0.04, p=0.02) **MIP-3α** (β= -0.09±0.04, p=0.04)
miR-320a	**K18 M30** (β= 0.06±0.03, p=0.04) **Alkaline Phos.** (β= 0.18±0.06, p=0.002)	**HOMA-β** (β= -0.04±0.02, p=0.04) **TNFα** (β= 0.09±0.04, p=0.01) **IL6** (β= 0.05±0.03, p=0.04) **Hyaluronic acid** (β= 0.05±0.02, p=0.048) **Endotoxin** (β= -0.04±0.02, p=0.049) **Adiponectin** (β= 0.06±0.03, p=0.02) **Resistin** (β= 0.12±0.03, p=0.0004) **MIP-3α** (β= 0.09±0.04, p=0.02)

CONCLUSIONS

- The previously reported high prevalence of K-18 categorized liver disease (predominantly necrotic) observed in ACHS-I (PMID:29684222) was confirmed in the follow-up ACHS-II study.
- Consistent with hepatotoxicity, well-validated alternate indicators of liver injury (e.g., ALT and miR-122-5p) were significantly increased in the groups with K18-categorized liver disease. They were increased to a greater degree in the apoptosis *vs.* the necrosis group.
- Key associations between miRs (e.g., miR-122-5p and miR-192-5p) and liver disease category which were recently reported in ACHS-I (PMID:34989596) were confirmed in ACHS-II.
- By complete linkage analysis, serum liver toxicity biomarkers (e.g., miRs, K18, and routine biochemistries) clustered into four groups associated with hepatic steatosis (G2), hepatitis (G4), cirrhosis (G4), liver hyperplasia (G1-G2), and hepatocellular carcinoma (G1-G3). Therefore, circulating miRs appear to provide additional non-overlapping information to routine biochemistries.
- Several hepatotoxicity miRs (most notably, miR-29-3p (total dioxin TEQ and PCB TEQs), miR-185-5p (Total dioxinTEQ), and miR-320a (PCDD TEQ and PCDF TEQ) were associated with biomarkers of aryl hydrocarbon receptor activation by environmental pollutants. PCDF TEQ was associated with greatest number of liver toxicity biomarkers. Albumin was inversely associated with almost all TEQ biomarkers tested.
- miR-29-3p, miR-185-5p and miR-320a were in turn significantly associated with biomarkers of intermediary metabolism (lipid, carbohydrate and protein) and adipocytokines.
- We postulate that serum miRs could serve as accessible biomarkers of both dioxin-induced metabolic toxicity, liver disease, and/or adaptation.
- Reverse causality cannot be excluded.

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