TOTAL DIOXIN TOXIC EQUIVALENCY AND SEX ARE ASSOCIATED WITH BIOMARKERS OF HEPATIC LIPID METABOLISM, INFLAMMATION, FIBROSIS, AND FUNCTION IN A SUBSET OF ACHS-II PARTICIPANTS

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Introduction. Exposure to persistent organic pollutants including polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs) have been associated with multiple disease endpoints such as liver disease, diabetes and cardiovascular disorders [1, 2]. Mechanistically, PCBs, PCDDs, and PCDFs act as metabolism-disrupting and endocrine-disrupting chemicals (MDCs & EDCs) through activation of hepatic and hormone receptors including the aryl hydrocarbon receptor (AhR); thereby increasing susceptibility to metabolic diseases such as non-alcoholic fatty liver disease (NAFLD) [3, 4]. Numerous cohort studies have reported positive correlations between serum PCB levels and NAFLD [5-9]. Importantly, NAFLD and its more advanced form, nonalcoholic steatohepatitis (NASH), are currently the most common forms of liver disease worldwide with a global prevalence of over 25% [10]. NAFLD is a spectrum of disorders in the liver ranging from lipid accumulation (steatosis), often accompanied by inflammation and hepatocyte death (steatohepatitis), and may progress to cirrhosis, liver failure and liver-related mortality [11]. Key mechanisms that dictate NAFLD development and progression include dysregulated lipid and carbohydrate metabolism, leading to dyslipidemia, steatosis and altered glucose levels [4]; increased Kupffer cell-derived pro-inflammatory cytokines including tumor necrosis factors alpha (TNFα), indicative of inflammation [2]; increased Stellate cell-derived hyaluronic acid, indicative of fibrosis [12]; increased cytokeratin 18 (CK 18 M65) levels, indicative of hepatocyte death [13]; and decline in hepatic function such as decreased albumin production [14]. Previously, our group demonstrated the high prevalence of suspected NAFLD in Phase I participants of the Anniston Community Health Survey (ACHS) [9]. Liver disease was associated with exposures to multiple mono-ortho PCB congeners. In the follow-up study, ACHS-II, additional chemicals were measured, such as, non-ortho PCBs, PCDDs, and PCDFs. At the 2017 Dioxin Meeting, we reported the high prevalence of fatty liver disease in ACHS-II participants and reported associations between dioxin-like chemicals with mechanistic, serological biomarkers of NAFLD. The purpose of the current study is to analyze a subset of ACHS-II participants who demonstrated abnormal CK 18 levels (suspected liver disease), and determine associations between *i*) total dioxin toxic equivalency (TEQ), or *ii*) sex and serological biomarkers of liver disease.

Materials and Methods. The study design of ACHS-II was as previously reported. Informed consent was obtained from all participants. Analysis of de-identified archived serum samples and data was approved by the University of Louisville Institutional Review Board. Serologic exposure biomarkers were measured. Disease biomarkers were measured by either ELISA or by multi-analyte chemiluminescent detection. Two hundred nine (209; 62%) ACHS II study participants with having abnormal CK18 indicated liver disease (CK18 M65>300 U/ML) were included in the present analyses. CK18 has been proposed to be a NAFLD biomarker because it correlates with histology [13]. Total dioxin TEQ (wet weight, ww) was derived by summing levels of non-ortho PCB TEQ, PCDD TEQ, and PCDF TEQ. The TEQ for an individual congener was determined for each participant by multiplying that congener's wet weight concentration by its World Health Organization toxic equivalency factor (TEF). Descriptive statistics were used to summarize demographic and exposure characteristics stratified by low and high total dioxin TEQ exposure based on the median (100 pg/g). Differences in characteristics were assessed using *t*-test for continuous variables or chi-square tests for categorical variables. All exposure variables were $\log_e$-transformed in the tests and presented in raw values. Biomarkers ($\log_e$-transformed) of interest were assessed for their joint association with total dioxin TEQ (low/high) and sex (male/female) using a linear model. Based on differences in patient demographics, these models are adjusted for age, race, and history of diabetes. Analyses were performed using SAS (SASv9.4, Cary, NC, USA). Significance was set at 0.05 and *P*-values were corrected for multiple comparisons using the Holm-Bonferonni method.

Results and Discussion. Demographic characteristics of 209 ACHS-II participants with suspected liver disease (abnormal CK18) are presented in Table 1. Total dioxin TEQ in our sample of 209 ACHS-II participants was stratified by median (100 pg/g; mean=144 pg/g). Those with high total dioxin TEQ tended to be older (68 y *vs.* 56 y, $p<0.001$) and were more likely to be female (84% *vs.* 52%, $p<0.001$), African-American (52% *vs.* 37%, $p=0.02$), and have a history of diabetes (53% *vs.* 34%, $p=0.004$). No differences in BMI and drinking or smoking statuses were observed.

Table 1. Demographic characteristics in abnormal CK18 liver disease[a] stratified by total dioxin TEQ (ww), ACHS-II Study (N=209)

Characteristic	Total TEQ (ww)[b]				*P*-value	Total (N=209)	
	Low (N=104)		High (N=105)				
	Mean	SD	Mean	SD		Mean	SD
Age (years)	56.3	12.9	68.1	10.5	**<0.001**	62.2	13.2
BMI (kg/m^2)	32.0	7.7	30.8	6.6	0.22	31.4	7.2
Total Dioxin TEQ (pg/g ww)	58.7	23.8	227.9	172.3	**<0.001**	143.7	149.4
Non-ortho PCB TEQ (ww)	9.5	7.7	84.6	118.2	**<0.001**	47.2	91.8
PCDD TEQ (pg/g ww)	34.9	15.3	91.2	43.1	**<0.001**	63.2	42.9
PCDF TEQ (pg/g ww)	9.6	4.6	23.2	17.9	**<0.001**	16.4	14.7
	N	%	N	%		N	%
Sex					**<0.001**		
Male	50	48.1	17	16.2		67	32.1
Female	54	51.9	88	83.8		142	67.9
Race/ethnicity					**0.02**		
Non-Hispanic White	66	63.5	50	47.6		116	55.5
African-American	38	36.5	55	52.4		93	44.5
Diabetes Status					**0.004**		
Ever Diabetic	35	33.7	56	53.3		91	45.5
Non-diabetic	69	66.3	49	46.7		118	56.5
Typical Number of Drinks Per Week in Past 12 Months					0.92		
No drinks	72	69.2	74	70.5		146	69.9
Within defined limits[c]	22	21.2	20	19.0		42	20.1
More than limit	10	9.6	11	10.5		21	10.0
Current Smoker					0.30		
No	78	75.0	85	81.0		163	78.0
Yes	26	25.0	20	19.0		46	22.0

Abbreviations: ACHS-II, Anniston Community Health Study Phase 2; BMI, body mass index; CK, cytokeratin; g, grams; kg, kilograms; m, meter; PCB, polychlorinated biphenyl; PCDD, polychlorinated dibenzodioxins; PCDF, polychlorinated dibenzofurans; pg, picograms; TEQ, toxicant-equivalent; ww, whole weight.

Note: Not all percentages add to 100% due to rounding. Values are presented as N (%) or Mean±SD.

[a] Abnormal CK18 liver disease is defined as having TASH (N=154; CK18 M30<200 and CK18 M65<300) or any other liver disease (N=55; CK18 M30>200). [b] Lower TEQ: Total TEQ (ww) < 100 (pg/g); Higher: Total TEQ (ww) > 100 (pg/g). [c] Limits are <= 7 drinks for females and <= 14 drinks for males.

Next, we investigated associations between TEQ and liver disease biomarkers in the 209 participants. In models without adjustment (Table 2), high total dioxin TEQ was positively associated with very low density lipoprotein

(VLDL), TNFα, and hyaluronic acid and negatively associated with albumin. The association with male sex was positively associated with VLDL and albumin (p<0.05 for all). After adjusting p-values, associations between high total dioxin TEQ and TNFα (p_{adj}=0.047) and hyaluronic acid (p_{adj} <0.001) remained.

Table 2. Unadjusted joint association of total dioxin TEQ (whole weight) exposure and sex with primary biomarkers in ACHS II participants with abnormal CK18 liver disease[a], ACHS II Study (N=209).

	Unadjusted Model							
	High total dioxin TEQ (ww)[b]				**Male**			
Biomarker	**Beta**	**SE**	**P**	**P-adj**	**Beta**	**SE**	**P**	**P-adj**
Lipid Metabolism: VLDL	0.32	0.07	**<.0001**	**0.003**	0.21	0.0	**0.01**	0.08
Glucose Metabolism: HOMA-B	-0.31	0.16	0.0501	0.30	-0.14	0.1	0.41	>0.99
Inflammation: TNFα	0.32	0.11	**0.005**	**0.047**	0.23	0.1	0.06	0.30
Fibrosis: Hyaluronic Acid	0.65	0.13	**<.0001**	**<.000**	0.25	0.1	0.08	0.32
Liver Function: Albumin	-0.03	0.01	**0.02**	0.15	0.04	0.0	**0.02**	0.15
Liver Cell Death: CK18 M65	-0.04	0.05	0.44	>0.99	0.01	0.0	0.81	>0.99

Adjusted P-values are corrected for multiple comparisons using Holm-Bonferroni method.
Abbreviations: ACHS II, Anniston Community Health Study Phase 2; adj, adjusted; CK 18, cytokeratin 18; HOMA, homeostatic model assessment; ml, milliliters; ng. nanograms; P, p-value; SE, standard error; TEQ, toxicant-equivalent; TNF, tumor necrosis factor; VLDL, very-low-density lipoprotein.
[a] Abnormal CK18 liver disease is defined as having TASH (N=154; CK18 M30<200 and CK18 M65<300) or any other liver disease (N=55; CK18 M30>200). [b] Lower TEQ: Total TEQ (ww) < 100 (pg/g); Higher: Total TEQ (ww) > 100 (pg/g).

When adjusted, high total dioxin TEQ was not associated with any biomarker, except albumin in lipid-adjusted models (estimate=-0.05, p =0.01, p_{adj} =0.06). However, we did observe persistent sex associations with VLDL and albumin in models with and without lipid adjustment (data not shown). All models have similar direction and strength of association. We further observe that the direction of both the high total dioxin TEQ and male sex estimates are similar in our selected biomarkers, with the exception of CK18 M65 and albumin; however, the interaction of these variables is insignificant, even though both differ significantly by sex, due to the limited number of males in the high total dioxin TEQ category (n=17).

Mechanistic biomarkers for lipid metabolism, inflammation, fibrosis and liver function were associated with high total dioxin TEQ, implicating the role of AhR activation by environmental chemicals as a potential mediator for liver disease development and progression. In addition, the results suggested that sex plays another crucial role in determining NAFLD outcomes such as impaired liver function. Future investigations looking at sex differences in ACHS participants are needed. Likewise, the potential role of race/ethnicity on toxicant associated fatty liver diseases warrants future investigation. An important limitation to address is the lack of liver biopsies needed to confirm liver disease diagnosis in this population. To address this limitation, stronger statistical models for NAFLD assessment in this population are currently being investigated by our group. The current results confirmed our previously reported findings that dioxin-like chemicals are associated with mechanistic fatty liver disease in ACHS-II participants. Furthermore, given these findings, the impact of environmental dioxin exposures on liver pathology in NAFLD requires future investigation.

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