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Research Article

Liver Disease in a Residential Cohort With Elevated Polychlorinated Biphenyl Exposures

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ABSTRACT

Endocrine and metabolism disrupting chemicals (EDCs/MDCs) have been associated with environmental liver diseases including toxicant-associated steatohepatitis (TASH). TASH has previously been characterized by hepatocellular necrosis, disrupted intermediary metabolism, and liver inflammation. Polychlorinated biphenyls (PCBs) are environmental EDCs/MDCs associated with the genesis and progression of steatohepatitis in animal models and human liver injury in epidemiology studies. The cross-sectional Anniston Community Health Survey (ACHS) investigates ortho-substituted PCB exposures and health effects near a former PCB manufacturing complex. The rates of obesity, diabetes, and dyslipidemia were previously determined to be high in ACHS. In this study, 738 ACHS participants were categorized by liver disease status using the serum cytokeratin 18 biomarker. Associations between PCB exposures and mechanistic biomarkers of intermediary metabolism, inflammation, and hepatocyte death were determined. The liver disease prevalence was high (60.2%), and 80.7% of these individuals were categorized as having TASH. Sex and race/ethnicity differences were noted. TASH was associated with increased exposures to specific PCB congeners, insulin resistance, dyslipidemia, proinflammatory cytokines, and liver necrosis. These findings are consistent with PCB-related steatohepatitis. ΣPCBs was inversely associated with insulin resistance/production, leptin, and hepatocyte apoptosis, while other adipocytokines were increased. This is possibly the largest environmental liver disease study applying mechanistic biomarkers ever performed and the most comprehensive analysis of PCBs and adipocytokines. It provides insight into the mechanisms of PCB-related

endocrine and metabolic disruption in liver disease and diabetes. In the future, associations between additional exposures and liver disease biomarkers will be evaluated in the ACHS and follow-up ACHS-II studies.

Key words: PCB; toxicant-associated steatohepatitis; TASH; NASH; endocrine disrupting chemicals; metabolism disrupting chemicals.

Liver injury and disease may occur as a consequence of industrial chemical exposures (Cave *et al.*, 2011). Steatohepatitis is a form of liver disease characterized by disrupted intermediary metabolism (including insulin resistance), proinflammatory cytokine elevation, and hepatocyte death. Steatohepatitis was initially associated with alcohol abuse (alcoholic steatohepatitis, ASH) and later with obesity/metabolic syndrome (nonalcoholic steatohepatitis [NASH]; Joshi-Barve *et al.*, 2015). Toxicant-associated steatohepatitis (TASH) occurs as a result of exposures to endocrine and metabolism disrupting chemicals (EDCs/MDCs) (Cave *et al.*, 2010b; Heindel *et al.*, 2017), and differs from other etiologies by hepatocyte death mechanism: primarily necrosis in TASH and apoptosis in ASH/NASH (Joshi-Barve *et al.*, 2015; Wahlang *et al.*, 2013a). EDCs are defined as chemicals or mixtures that interfere with any aspect of hormone action (Zoeller *et al.*, 2012). MDCs promote metabolic disease states including fatty liver, obesity, hyperlipidemia, and diabetes, but may require a "second hit" such as increased dietary sugar or fat (Heindel *et al.*, 2015, 2017). The global prevalence of nonalcoholic fatty liver disease (NAFLD, inclusive of NASH) is 25.2% (Younossi *et al.*, 2016). The metabolic conditions most frequently associated with NAFLD are: hyperlipidemia (69.16%), obesity (51.3%), and diabetes (22.5%). These same metabolic comorbidities have also been associated with EDC/MDC exposures.

Polychlorinated biphenyls (PCBs) are persistent organic pollutants (POPs) and EDCs/MDCs. There are 209 possible congeners based on biphenyl ring chlorination pattern. Structurally, PCB congeners may be classified as either ortho-substituted or nonortho-substituted (coplanar). PCB mixtures were used in a variety of industrial applications before they were banned by the Stockholm Convention on POPs. Because PCBs are lipophilic and bio-degrade slowly, they have bio-accumulated in the food chain. PCBs were present in 100% of adult National Health and Nutrition Examination Survey (NHANES) 2003–04 participants (Cave *et al.*, 2010a). In NHANES, the methods of Clark *et al.* (2003) were used to identify a subgroup with "unexplained" ALT elevation consistent with NAFLD; and associations between PCB exposures and suspected NAFLD were then determined (Cave *et al.*, 2010a). PCB exposures were dose-dependently associated with suspected fatty liver disease. The epidemiological association between PCBs and liver injury was subsequently confirmed by other laboratories (Kim *et al.*, 2010; Kumar *et al.*, 2014a; Rantakokko *et al.*, 2015; Serdar *et al.*, 2014; Yorita Christensen *et al.*, 2013). However, liver disease mechanisms were not investigated in these studies. A causal relation between PCBs and fatty liver disease has been well-documented in rodent studies (Al-Eryani *et al.*, 2015; Gadupudi *et al.*, 2016; Hennig *et al.*, 2005; Kaiser *et al.*, 2012; Wahlang *et al.*, 2013b, 2014b). High-dose coplanar PCBs can cause steatohepatitis (Gadupudi *et al.*, 2016). Other PCBs administered to diet-induced obesity mouse models were "second hits" which worsened steatosis or mediated the progression of steatosis to steatohepatitis (Wahlang *et al.*, 2013b, 2014b).

Like other EDCs/MDCs, PCB effects have been attributed to receptor interactions. PCB structure determines receptor specificity and biological mechanism. Coplanar PCBs potently activate the aryl hydrocarbon receptor (AhR). Both coplanar and ortho-substituted PCBs interact with hepatic nuclear receptors (Gadupudi *et al.*, 2016; Wahlang *et al.*, 2016). PCBs can ligand-activate some human nuclear receptors such as the constitutive androstane receptor (CAR) and the pregnane X receptor (PXR) (Wahlang *et al.*, 2014a). Other effects appear to be indirect, involving phosphorylation-dependent receptor activation by xenobiotic-responsive cell surface receptors, and transcriptional control of receptor expression (Gadupudi *et al.*, 2016; Hardesty *et al.*, 2016; Joshi-Barve *et al.*, 2015; Wahlang *et al.*, 2013a, 2014a). Nuclear receptors and the AhR regulate hepatic xenobiotic/intermediary metabolism and the inflammatory response. Not surprisingly, these receptors have been implicated in steatohepatitis pathogenesis (Cave *et al.*, 2016). Indeed, in a mouse model of PCB-induced TASH, nuclear receptors modulated PCB-dependent changes in energy metabolism, behavior, and inflammation (Wahlang *et al.*, 2016).

Aroclor PCB mixtures were produced commercially from 1935 to 1971 in Anniston, Alabama. Chemical releases resulted in PCB contamination of soil, ground water and some residents, prompting the initiation of the Anniston Community Health Survey (ACHS). This cross-sectional residential cohort study has been studied to determine associations between ortho-substituted PCBs and health-related outcomes (Aminov *et al.*, 2013; Goncharov *et al.*, 2010; Pavuk *et al.*, 2014a; Silverstone *et al.*, 2012). Key findings include: increased mean PCB levels compared with NHANES (2-3 fold) (Pavuk *et al.*, 2014a); a high prevalence of obesity (54%) (Pavuk *et al.*, 2014b); and associations between PCB exposures and diabetes (Silverstone *et al.*, 2012) and dyslipidemia (Aminov *et al.*, 2013). Given the high burden of these metabolic conditions previously associated with both steatohepatitis and EDC/MDC exposures, we investigated mechanistic liver disease/injury biomarkers in archived ACHS serum samples. We hypothesized that there would be a high prevalence of necrotic liver disease associated with PCB exposures, proinflammatory cytokines, and altered intermediary metabolism—consistent with TASH.

MATERIALS AND METHODS

Study design and serum ortho-substituted PCB measurement. The ACHS study design has been previously described in Aminov *et al.* (2013), Goncharov *et al.* (2010), Pavuk *et al.* (2014a), and Silverstone *et al.* (2012). Briefly, in the 2-stage sampling procedure, 3320 Anniston, AL households were randomly selected. West Anniston households, located closer to the former PCB production facility, were oversampled. All selected residences were visited by ACHS staff, who made contact with an individual from 1823 of the targeted households. Of these, 1110 consented to participate, and 1 adult (>18 years of age) from each consenting household was selected to complete the survey, resulting in an overall completion rate of 61%. The 738 subjects included in this analysis of stored serum previously completed a survey; clinic visit; fasting blood draw with determination of serum glucose, lipids, and PCBs; and had sufficient remaining archived serum (−80°C) for liver biomarker measurement.

Serum concentrations of 35 ortho-substituted PCB congeners were determined by high-resolution gas chromatography/isotope dilution high-resolution mass spectrometry at the National Center for Environmental Health Laboratory of the Centers for Disease Control and Prevention (Atlanta, Georgia) (Aminov *et al.*, 2013; Goncharov *et al.*, 2010; Pavuk *et al.*, 2014a; Silverstone *et al.*, 2012). The previously collected data and archived serum samples were provided to the Cave Laboratory in a de-identified fashion. The University of Louisville Institutional Review Board approved this research.

Measurement of mechanistic liver disease biomarkers. Biomarkers of hepatocyte death, hepatic intermediary metabolism, and adipocytokines were measured in archived serum samples. Cytokeratin 18 (CK18) M65 and M30 (PEVIVA 10020 and 10010, Diapharma, Cincinnati, Ohio) were measured by separate enzyme-linked immunosorbent (ELISA) assays, each using a monoclonal antibody recognizing a different CK18 epitope. CK18 is a structural protein located in cells of epithelial origin. CK18 is enriched in hepatocytes, and can be detected in both whole and caspase-cleaved forms, allowing for the differentiation of apoptosis (caspase-cleaved CK18 or M30) from total cell death (CK18 M65). CK18 was selected instead of ALT as the hepatocyte death biomarker for several reasons. First, preliminary studies indicate that CK18 provides increased sensitivity over ALT for environmental liver diseases (Cave *et al.*, 2010b, 2011) and other chronic liver diseases such as NASH (Feldstein *et al.*, 2009). Second, the pattern of CK18 elevation is reflective of the hepatocyte cell death mechanism (apoptosis *vs*. necrosis); and CK18 may be used to non-invasively categorize subtypes of liver disease (Cave *et al.*, 2010b, 2011; Joshi-Barve *et al.*, 2015; Wahlang *et al.*, 2013a). Third, CK18 is currently the most extensively validated serum biomarker for steatohepatitis as a stand-alone test correlating with histology (Castera, 2015; Feldstein *et al.*, 2009); and the inclusion of transaminases with CK18 in order to create a prediction model did not improve the diagnostic value of CK18 alone in NASH (Feldstein *et al.*, 2009). Adipocytokines were measured using 2 separate multiplex bead arrays run on a Luminex IS100 system (EMD Millipore Corporation, Billerica, Massachusetts). The first array (HADK2MAG-61 K) measured interleukin 6 (IL-6), interleukin 8 (IL-8), monocyte chemotactic protein-1 (MCP-1), tumor necrosis factor α (TNFα), interleukin 1-β (IL-1β), insulin, and leptin. For the second array (HADK1MAG-61 K), samples were diluted 1:400 in the provided assay buffer, and adiponectin, resistin, and total plasminogen activator inhibitor-1 (PAI-1) were measured. The homeostatic models of assessment (HOMA) (Wallace *et al.*, 2004) were used to evaluate insulin resistance (HOMA-IR) and pancreatic beta cell function (HOMA-B).

Data analysis and procedures. Serum CK18 M30 and M65 were used to categorize subjects by liver disease status based on previously published procedures and cutoffs (Cave *et al.*, 2011; Feldstein *et al.*, 2009). These categories included no evident liver disease (None, M30 $<$ 200 U/L and M65 $<$ 300 U/L); TASH (M30 $<$ 200 U/L and M65 $>$ 300 U/L, consistent with necrosis); and other liver disease (Other, M30 $>$ 200 U/L, consistent with apoptosis). Because liver biopsies were not available, categorization procedures were internally validated by subsequently determining the possible associations between liver disease category with biomarkers of intermediary metabolism and inflammation. Based on the literature, the TASH category should be associated with each of these broad disease mechanisms.

Counts and percentages were calculated for the main predictors for the ACHS population and by liver disease status. Differences in means and frequencies by liver disease status were tested with a 1-way ANOVA or chi-square test, respectively. The TASH and Other liver disease categories were always compared against None. Biomarkers were analyzed with multivariable generalized linear models to assess their relation to serum wet weight PCB levels with or without confounders. Adjusted multinomial and regression models were constructed to determine possible associations between individual PCB ortho-substituted congeners with liver disease status and CK18. Univariate regression analysis was performed to determine associations between ΣPCBs and demographic variables or the CK18 liver injury biomarkers. Unless noted elsewhere, all analyses were adjusted for age (years; continuous), body mass index (BMI, kg/m^2; continuous), gender (male vs female), race (African-American vs nonHispanic white), diabetes status (none, prediabetic, or diabetic), alcohol use, and total lipid levels. Biomarkers, PCB levels, and total lipid levels were log-transformed in the models and back-transformed to standard units in tables and figures. Biomarkers below the level of detection (IL-1β $<$ 1.3 pg/ml; IL-6 $<$ 0.96 pg/ml; insulin $<$ 9.6 pg/ml; IL-8 $<$ 0.64 pg/ml; MCP-1 $<$ 1.3 pg/ml; TNFα $<$ 0.64 pg/ml) were set to half the lower limit of detection. Leptin levels greater than the upper limit of quantification (leptin $>$ 600 ng/ml, 1 record) were set to 600 ng/ml. For individual congeners as well as ΣPCBs, previously established analysis and reporting conventions were used (Goncharov *et al.*, 2010; Silverstone *et al.*, 2012). PCB levels reported by the laboratory as "undetectable" were substituted with a value equal to the lower limit of detection for the specific congener divided by the square root of 2 (Hornung and Reed, 1990). When measured PCB concentrations below the stated lower limit of detection were reported from the laboratory, those measurements (rather than substituted values) were used (Pavuk *et al.*, 2014a). Values for total lipids were calculated by the formula Total Lipids = (2.27 * Total Cholesterol) + Triglycerides + 62.3 mg/dL as previously described. This method was previously employed to analyze and report associations between serum PCB level and diabetes in ACHS (Silverstone *et al.*, 2012). Regression lines were plotted for each category of liver disease status. Statistical analyses were performed with SAS version 9.4 (SAS Institute, Cary, North Carolina). A statistical significance level of 0.05 was used.

RESULTS

Demographic Information and Liver Disease Prevalence

Population demographics are provided in Table 1. The 738-subject cohort was comprised of a high percentage of African-Americans (46.8%), females (70.1%), subjects ≥50-years old (63.0%), nondrinkers (70.6%), and nonsmokers (69.0%). Most participants (80.3%) were either overweight (BMI ≥ 25 kg/m^2) or obese (BMI ≥ 30 kg/m^2). Liver disease prevalence was high (60.2% overall, 48.6% TASH, and 11.5% with Other), as predicted by animal models of diet-induced obesity and PCB co-exposures (Wahlang *et al.*, 2013b, 2014b). Most (80.9%) of the suspected liver disease cases were categorized as TASH. Significant intergroup differences were seen with respect to race/ethnicity, gender, and age. The overall liver disease prevalence was high in nonHispanic whites (70.2%). NonHispanic whites comprised a significantly higher percentage of the TASH category (62.1%) versus the None category (39.8%, $p < .05$). Likewise, the overall liver disease prevalence was also high in males (67.4%). Males

Table 1. Demographic Characteristics by Liver Disease Status

Characteristic	Liver Disease Status			*p*-value	Total ($n = 738$)
	None ($n = 294$)	TASH ($n = 359$)	Other ($n = 85$)		
Age (years)	54.1 ± 15.7	56.0 ± 16.3[a]	51.5 ± 15.1	**.04**	54.7 ± 15.9
BMI (kg/m^2)	31.5 ± 7.8	30.9 ± 7.7	32.1 ± 7.7	.34	31.25 ± 7.7
Gender				**.03**	
Male	72 (24.5)	123[b] (34.3)	26 (30.6)		221 (30.0)
Female	222 (75.5)	236 (65.7)	59 (69.4)		517 (70.1)
Race/ethnicity				**<.001**	
NonHispanic White	117 (39.8)	223[b] (62.1)	53[c] (62.4)		393 (53.3)
African/American	177 (60.2)	136 (37.9)	32 (37.7)		345 (46.8)
Age (years)				.13	
<30	24 (8.2)	31 (8.7)	8 (9.4)		63 (8.5)
30–40	41 (14.0)	25 (7.0)	12 (14.1)		78 (10.6)
40–50	45 (15.3)	69 (19.2)	18 (21.2)		132 (17.9)
50–60	66 (22.5)	75 (20.9)	20 (23.5)		161 (21.8)
60–70	63 (21.4)	75 (20.9)	15 (17.7)		153 (20.7)
≥70	55 (18.7)	84 (23.4)	12 (14.1)		151 (20.5)
BMI (kg/m^2)				.40	
<18.5	3 (1.0)	2 (0.6)	0 (0.0)		5 (0.7)
18.5–24.9	61 (20.8)	67 (18.7)	14 (16.5)		142 (19.3)
25–29.9	62 (21.1)	109 (30.4)	20 (23.5)		191 (26.0)
30–34.9	77 (26.2)	90 (25.1)	22 (25.9)		189 (25.7)
35–39.9	48 (16.3)	48 (13.4)	15 (17.7)		111 (15.1)
≥40	42 (14.3)	42 (11.7)	14 (16.5)		98 (13.3)
Missing	1 (0.3)	1 (0.3)	0 (0.0)		2 (0.3)
Number of drinks in last 30 days				.70	
No drinks	205 (69.7)	254 (70.8)	62 (72.9)		521 (70.6)
Within defined limits[d]	63 (21.4)	73 (20.3)	14 (16.5)		150 (20.3)
More than limit	16 (5.4)	23 (6.4)	8 (9.4)		47 (6.4)
Missing	10 (3.4)	9 (2.5)	1 (1.2)		20 (2.7)
Current Smoker				.69	
No	208 (70.8)	243 (67.7)	58 (68.2)		509 (69.0)
Yes	86 (29.3)	116 (32.3)	27 (31.8)		229 (31.0)
West Anniston resident				.25	
No	43 (14.6)	62 (17.3)	12 (14.1)		117 (15.9)
Yes	251 (85.4)	297 (82.7)	73 (85.9)		621 (84.2)
CK18 M65 (U/l)	233.6 ± 42.6	430.6 ± 122.1[a,b]	792.5 ± 584.9[c]	**<.001**	393.8 ± 276.0
CK18 M30 (U/l)	97.9 ± 22.0	124.0 ± 28.2[a,b]	407.6 ± 324.6[c]	**<.001**	146.3 ± 147.1

Liver disease categories were defined by serum CK18 M65 and M30 values as previously published in Cave *et al.* (2011): "None" M65 < 300 U/l and M30 < 200 U/l); "TASH" M65 > 300 U/l and M30 < 200 U/l; and "Other" M30 > 200 U/l.

Data are *n* (%) or mean ± SD. Not all percents add to 100% due to rounding.

A *p*-value is 1-way ANOVA (means) or Pearson chi-square test, across liver disease categories.

[a]Adj-$p \leq .05$ in pair-wise comparison of TASH versus Other liver disease category.

[b]Adj-$p \leq .05$ in pair-wise comparison of None versus TASH liver disease category.

[c]Adj-$p \leq .05$ in pair-wise comparison of None versus Other liver disease category.

[d]Limits are ≤ 30 drinks for females and ≤60 drinks for males.

The values in bold are those that were statistically significant.

comprised a significantly higher percentage of the TASH category (34.3%) versus the None category (30.6%, $p < .05$). These results are consistent with previous reports documenting increased fatty liver susceptibility in males and Caucasians (Pan and Fallon, 2014). TASH subjects had a higher mean age than Other (56.0 ± 16.3 vs 51.5 ± 15.1, $p < .05$). No intergroup differences were seen in other demographic variables including West Anniston residence, BMI, alcohol consumption, or smoking status. The rate of significant alcohol consumption (≥30 drinks in the last 30 days for females or ≥60 drinks in the last 30 days for males) was low (6.4%) and cannot explain the high rate of liver disease observed in this cohort.

CK18 is a biomarker of liver injury (hepatocyte death) and disease. Mean CK18 M65 was significantly higher in TASH (430.6 ± 122.1 U/l) and Other (792.5 ± 584.9) versus None (233.6 ± 42.6, $p < .05$). In both the liver disease groups, these values were well above the M65 cutoff value of 300 U/l. Likewise, mean CK18 M30 was significantly higher in TASH (124.0 ± 28.2 U/l) and Other (407.6 ± 324.6) versus None (97.9 ± 22.0, $p < .05$). CK18 M30 in the Other group was approximately 2-fold higher than its cutoff value (200 U/l), but remained below its cutoff value in TASH based upon the categorization procedures. The mean CK18 elevations in TASH (M65) and Other (M65 and M30) are likely to be clinically significant, and are also reflective of different hepatocyte cell death mechanisms based on CK18 ratios. The mean M30: M65 ratio was 25% in the TASH category (consistent with predominantly nonapoptotic cell death, likely necrosis) and 51% in the Other category (consistent with slightly predominant apoptosis). The former value is consistent with prior human studies of TASH (26%–33%); while the latter is similar to that

Table 2. Adjusted Beta Coefficients of Significant Associations of Liver Disease Status and CK18 Biomarkers by Individual PCB Congeners

		Liver Disease[a]				M65[b]		M65[b]	
		TASH versus None							
		Estimate	*p*-value	Estimate	*p*-value	Estimate	*p*-value	Estimate	*p*-value
PCB Congener	28	0.24	**.03**					0.07	**<.001**
	44	0.45	**.04**	0.73	**.01**	0.11	**.01**	0.11	**.01**
	49	0.66	**.004**	0.71	**.01**	0.10	**.01**	0.10	**.01**
	52	0.37	**.01**	0.59	**.001**	0.09	**.001**	0.11	**<.001**
	66	0.29	**.002**					0.07	**<.001**
	101	0.20	**.05**					0.05	**.02**
	105							0.04	**.03**
	110	0.36	**.004**			0.05	**.04**	0.05	**.04**
	128	0.22	**.02**						
	149	0.24	**.02**						
	151	0.25	**.01**					0.05	**.01**
	172							0.04	**.02**
	178							0.04	**.03**
	187							0.04	**.04**
	195							0.04	**.04**

Liver disease categories were defined by serum CK18 M65 and M30 values as previously published in Cave *et al.* (2011): "None" M65 < 300 U/l and M30 < 200 U/l); "TASH" M65 > 300 U/l and M30 < 200 U/l; and "Other" M30 > 200 U/l.

Note that all congeners given in this table had at least a 96.7% detection rate. No significant differences were seen for PCB congeners 74, 87, 99, 118, 138, 146, 153, 156, 157, 167, 170, 177, 180, 183, 189, 194, 196, 199, 206, and 209.

[a]Multinomial model adjusted for age, sex, race, alcohol use, log lipids, diabetes (pre- and any vs none).

[b]Regression model adjusted for age, sex, race, alcohol use, log lipids, diabetes.

The values in bold are those that were statistically significant.

previously observed in NASH (55%–59%) (Cave *et al.*, 2010b, 2011). Mean CK18 M65 and M30 were both also significantly increased in Other versus TASH ($p < .05$). This is consistent with greater hepatocyte death in Other, although hepatocellular death was increased in both TASH and Other.

Associations Between PCBs, Liver Disease Categories, and Hepatocyte Death Mechanisms

In total 15 of the 35 measured congeners, were associated with a liver disease category and/or CK18 (Table 2). TASH was associated with 10 PCB congeners (PCBs 28, 44, 49, 52, 66 101, 110, 128, 149, and 151, $p < .05$), consistent with the concept that TASH is "toxicant-associated". Three congeners associated with TASH were also positively associated with Other (PCBs 44, 49, and 52, $p < .01$). In order to explore the possible relations between PCB levels and hepatocyte death and death mechanisms, associations between levels of congeners and CK18 M65 or M30 were determined. Thirteen congeners were positively associated with increased hepatocyte death as determined by M65 ($p < .05$). These included PCBs 105, 172, 178, 187, and 195 as well as the congeners associated with TASH (except for PCBs 128 and 149). Only 4 congeners (PCBs 44, 49, 52, and 110) were significantly associated with increased apoptosis as determined by CK18 M30. In summary, these data demonstrate increased hepatocyte death associated with exposures to 43% of the ortho-substituted PCBs tested. The predominant cell death mechanism associated with PCB exposures was necrosis.

M65 levels were higher in non-Hispanic whites ($p < .001$; Supplementary Table 1). ΣPCBs was not positively associated with either CK18 M65 or M30. This is not surprising because some, but not all, congeners were associated with increased liver cell death. A univariate sensitivity analysis excluding thirteen subjects with ΣPCBs beyond 4 SDs of the mean was performed (Supplementary Table 1). M65 levels were higher in non-Hispanic whites ($p < .001$) and in males ($p = .03$); while M30 was higher in nonHispanic whites ($p < .001$) and decreased with age ($p = .01$). An inverse association was found between ΣPCBs and M30 in this group, but not for the overall cohort. This suggests that ΣPCB exposure could decrease apoptosis.

Associations Between Liver Disease Categories or PCBs and Biomarkers of Intermediary Metabolism and Adipocytokines

Mean biomarker levels for the overall cohort and the 3 liver disease categories are presented in Table 3. The mean HOMA-IR for the overall cohort was elevated (4.8 ± 7.4) beyond one proposed cutoff for insulin resistance (HOMA-IR > 4.65 or HOMA-IR >3.60 and BMI >27.5 kg/m^2) (Stern *et al.*, 2005). This could be due to the high prevalence of suspected fatty liver disease, which has previously been associated with hepatic insulin resistance and diabetes (Younossi *et al.*, 2016). The TASH category, compared with None, was associated with significant differences in mean biomarkers of hepatic lipid metabolism (mean triglycerides, 147.0 ± 107.5 versus 119.3 ± 75.5 mg/dl, $p = .001$) and inflammation (MCP-1, 295.1 ± 244.4 vs 269.7 ± 175.2 pg/ml, $p = .009$; and PAI-1, 54.9 ± 21.4 vs 49.0 ± 19.4 ng/ml). The Other liver disease category, compared with None, was associated with increased mean triglycerides (160.6 ± 130.7 mg/dl vs 119.3 ± 75.5, $p = 0.002$); HOMA-IR (6.4 ± 8.3 vs 3.9 ± 5.4, $p = 0.017$); and PAI-1 (56.0 ± 23.3 ng/ml vs 49.0 ± 19.4, $p = .02$). Similar to HOMA-IR, mean insulin levels were significantly higher in Other than None. No differences were seen between groups with respect to mean IL-1β, IL-6, IL-8, resistin, TNFα, HOMA-B, low-density lipoprotein (LDL), adiponectin or leptin levels.

Associations between lipid-adjusted ΣPCBs with adipocytokines or intermediary metabolism biomarkers are given as beta coefficients with lipid-adjustment only (Table 4), or with adjustment for possible confounders (Table 5). Due to the lipid adjustment procedures, triglycerides, and LDL were not analyzed. In

Table 3. Biomarker Levels by Liver Disease Status

	Liver Disease Status				
	None ($n = 294$)	TASH ($n = 359$)	Other ($n = 85$)	p-value	Total ($n = 738$)
Adiponectin ng/ml					
Mean ± SD	17.2 ± 12.4	18.3 ± 19.1	15.1 ± 10.2	.22	17.5 ± 15.8
Glucose, mg/dl					
Mean ± SD	105.1 ± 40.4	108.2 ± 40.9	115.7 ± 53.9	.13	107.8 ± 42.5
HOMA-B					
Mean ± SD	164.2 ± 223.7	183.5 ± 334.3	219.6 ± 306.7	.29	180.0 ± 291.9
HOMA-IR					
Mean ± SD	3.9 ± 5.4	5.1 ± 8.4	6.4 ± 8.3[a]	**.01**	4.8 ± 7.4
IL-1β pg/ml					
Mean ± SD	2.4 ± 7.0	10.0 ± 76.4	13.1 ± 79.9	.17	7.3 ± 60.0
Not detectable	195	214	52		461
IL-6 pg/ml					
Mean ± SD	8.5 ± 42.0	16.4 ± 84.9	13.2 ± 40.6	.32	12.9 ± 66.4
Not detectable	35	23	4		62
IL-8 pg/ml					
Mean ± SD	78.7 ± 199.0	101.4 ± 257.4	86.7 ± 304.5	.49	90.7 ± 242.1
Not detectable	0	1	0		1
Insulin pg/ml					
Mean ± SD	492.8 ± 567.2	642.3 ± 1067.2	757.7 ± 918.7[a]	**.02**	596.0 ± 886.4
Not detectable	1	1	0		2
LDL, mg/dl					
Mean ± SD	117.0 ± 32.7	116.0 ± 39.8	116.5 ± 40.1	.94	116.5 ± 37.1
Leptin ng/ml					
Mean ± SD	30.5 ± 27.0	29.3 ± 39.7	26.1 ± 25.1	.57	29.4 ± 33.6
Not detectable	0	1	0		1
MCP-1 pg/ml					
Mean ± SD	269.7 ± 175.2	295.1 ± 244.4[b]	314.9 ± 193.6	**.01**	294.6 ± 194.1
Not detectable	0	1	0		1
PAI-1 ng/ml					
Mean ± SD	49.0 ± 19.4	54.9 ± 21.4[b]	56.0 ± 23.3[a]	**.001**	52.7 ± 21.1
Resistin ng/ml					
Mean ± SD	41.0 ± 31.7	44.1 ± 29.8	37.8 ± 19.1	.15	42.1 ± 29.6
TNFα pg/ml					
Mean ± SD	6.4 ± 5.5	9.2 ± 24.2	7.0 ± 7.1	.11	7.8 ± 17.4
Not detectable	3	2	0		5
Triglycerides, mg/dl					
Mean ± SD	119.3 ± 75.5	147.0 ± 107.5[b]	160.6 ± 130.7[a]	**.0002**	137.5 ± 100.3

Liver disease categories were defined by serum CK18 M65 and M30 values as previously published in Cave *et al*, (2011): "None" M65 < 300 U/l and M30 < 200 U/l); "TASH" M65 > 300 U/l and M30 < 200 U/l; and "Other" M30 > 200 U/lL.

p-value is 1-way ANOVA, across all liver disease categories, unadjusted.

[a]Adj-$p \leq .05$ in pair-wise comparison of None versus Other liver disease category.

[b]Adj-$p \leq .05$ in pair-wise comparison of None versus TASH liver disease category.

The values in bold are those that were statistically significant.

the model adjusted only for lipids (Table 4), TNFα ($p < .001$), IL-6 ($p = .001$), and adiponectin ($p = .01$) were positively associated with ΣPCBs. Insulin ($p = .03$) and HOMA-B ($p < 0.001$), which is a biomarker of pancreatic insulin production, were inversely associated with ΣPCBs. No associations were observed between ΣPCBs and IL-1β, IL-8, MCP-1, PAI-1, resistin, leptin, or HOMA-IR. In the fully-adjusted model, ΣPCBs was inversely associated with HOMA-IR ($p = .03$), insulin ($p = .04$), and leptin ($p < .001$) with a trend towards significance with respect to an inverse relationship with HOMA-B ($p = .08$) (Table 5). Regression curves presented in Figure 1 graphically depict the relationships between insulin and leptin levels with ΣPCBs. In order to investigate these associations further, adjusted beta coefficients for individual PCB congeners and these biomarkers were determined (Supplementary Table 2). Significant inverse associations were seen between 7 high molecular weight PCB congeners (PCBs 101, 157, 170, 180, 194, 196, and 199) and both HOMA-IR and insulin. Twenty-six congeners were inversely associated with leptin. Inverse associations were also found between HOMA-B and PCBs 180 and 194. Thus, depending on the model used, ΣPCBs was significantly associated with altered diabetes biomarkers (decreased HOMA-B, HOMA-IR, and insulin) and adipocytokines (decreased leptin and increased TNFα, IL-6, and adiponectin). The inverse associations between the PCBs and leptin or insulin/HOMA were particularly striking because they were present with up to 74% of the tested ortho-substituted congeners or for ΣPCBs in both models. The data confirm that PCBs are associated with endocrine disruption.

The TASH category, compared with None, was positively associated with increased adjusted beta coefficients for HOMA-IR ($p = .001$), HOMA-B ($p = .003$), insulin ($p = .01$), IL-1β ($p = .04$), IL-6 ($p = .03$), and PAI-1 ($p = .03$) (Table 5). The Other liver disease

Table 4. Biomarker levels by ΣPCBs[a]

	β	SE	*p*-value
Adiponectin	0.06	0.02	**.01**
Glucose	0.03	0.01	**<.001**
HOMA-B	−0.14	0.03	**<.001**
HOMA-IR	−0.03	0.03	.25
IL-1β	0.02	0.03	.55
IL-6	0.11	0.03	**.001**
IL-8	0.01	0.04	.80
Insulin	−0.06	0.03	**.03**
Leptin	0.02	0.03	.58
MCP-1	−0.02	0.02	.25
PAI-1	−0.01	0.01	.34
Resistin	−0.02	0.02	.24
TNFα	0.08	0.02	**<.001**

[a]Wet weight adjusted for natural log-transformed lipids.
The values in bold are those that were statistically significant.

category, compared with None, was associated with increased adjusted beta coefficients for HOMA-IR (p = .02), insulin (p = .04), and IL-6 (p = .01); and decreased leptin (p = .01). Importantly, the TASH category was associated with altered mean levels and/or adjusted beta coefficients for biomarkers of lipid metabolism (triglycerides), carbohydrate metabolism (HOMA-IR, HOMA-B, insulin), and inflammation (IL-1, IL-6, MCP-1, and PAI-1) (Tables 3 and 5). These associations increase the likelihood that subjects categorized by CK18 levels as TASH did indeed have steatohepatitis. This is because EDC/MDC-related steatohepatitis has previously been associated with abnormal hepatic lipid metabolism and with increased insulin resistance and proinflammatory cytokines (Heindel *et al.*, 2017). The associations between liver disease category or PCBs with biomarkers of intermediary metabolism and adipocytokines are summarized in Table 6.

DISCUSSION

With a sample size of 738 subjects, this report may be the largest environmental liver disease study performed to date using mechanistic biomarkers of liver disease (CK18 M30 and M65). The 60.2% (466/738) prevalence of liver disease is among the highest ever reported for a residential cohort. However, this prevalence estimate may be confounded by the application of the more sensitive CK18 biomarker as opposed to alanine aminotransferase (ALT), which has typically been used in other epidemiological studies investigating liver disease. 80.7% of liver disease cases (259 out of 466) were characterized by hepatocellular necrosis, a hallmark feature of TASH (Joshi-Barve *et al.*, 2015; Wahlang *et al.*, 2013a). The positive associations of individual PCB congeners with CK18 M65 elevation and the positive association of the TASH category with biomarkers of metabolic disease (elevated proinflammatory cytokines, insulin resistance, and hypertriglyceridemia) increase the likelihood that subjects categorized as TASH did, indeed, have PCB-related steatohepatitis. However, no imaging tests or biopsies were performed in this community-based study (not a clinical setting), and the demographic and exposure characteristics of the ACHS cohort are not representative of the overall population, limiting the generalizability of the study results. In this article, serologic biomarkers of liver injury/disease were reported. Ongoing Anniston studies investigate additional biomarkers of exposure and hepatic injury, morphology, fibrosis, and function.

Table 5. Adjusted[a] Beta Coefficients of Associations of ΣPCBs (Wet Weight) and Liver Status With Biomarkers

Cytokine/Adipokine	β	SE	*p*-value
Adiponectin			
PCB	0.01	0.03	.69
TASH versus None	0.04	0.05	.43
Other versus None	−0.06	0.08	.43
Glucose			
PCB	<0.001	0.01	1.00
TASH versus None	0.00	0.02	.90
Other versus None	0.03	0.03	.18
Insulin			
PCB	−0.07	0.04	**.04**
TASH versus None	0.22	0.06	**<.001**
Other versus None	0.19	0.09	**.04**
HOMA-B			
PCB	−0.07	0.04	0.08
TASH versus None	0.20	0.07	**.003**
Other versus None	0.13	0.10	.22
HOMA-IR			
PCB	−0.08	0.04	**.03**
TASH versus None	0.21	0.06	**.001**
Other versus None	0.21	0.09	**.02**
IL-1β			
PCB	0.01	0.05	.90
TASH versus None	0.18	0.09	**.04**
Other versus None	0.25	0.14	.07
IL-6			
PCB	0.05	0.05	.33
TASH versus None	0.27	0.09	**.003**
Other versus None	0.39	0.14	**.01**
IL-8			
PCB	0.08	0.07	.25
TASH versus None	0.15	0.12	.20
Other versus None	−0.04	0.18	.83
Leptin			
PCB	−0.14	0.04	**<.001**
TASH versus None	−0.06	0.06	.32
Other versus None	−0.24	0.09	**.01**
MCP-1			
PCB	0.02	0.03	.53
TASH versus None	0.01	0.05	.91
Other versus None	−0.07	0.08	.36
PAI-1			
PCB	−0.01	0.02	.51
TASH versus None	0.07	0.03	**.03**
Other versus None	0.07	0.05	.15
Resistin			
PCB	0.01	0.03	.81
TASH versus None	0.06	0.05	.20
Other versus None	−0.06	0.07	.41
TNFα			
PCB	0.04	0.03	.26
TASH versus None	0.07	0.06	.20
Other versus None	−0.04	0.09	.65

Liver disease categories were defined by serum CK18 M65 and M30 values as previously published in Cave *et al.* (2011): "None" M65 < 300 U/l and M30 < 200 U/l); "TASH" M65 > 300 U/l and M30 < 200 U/l; and "Other" M30 > 200 U/l.
[a]Adjustments were made for lipid levels, age, BMI, gender, race/ethnicity, diabetes, and alcohol use.
The values in bold are those that were statistically significant.

Transaminases will be included in these studies. The longitudinal follow-up study, ACHS-II, enables determination of the potential reversibility of TASH. The results of these ongoing

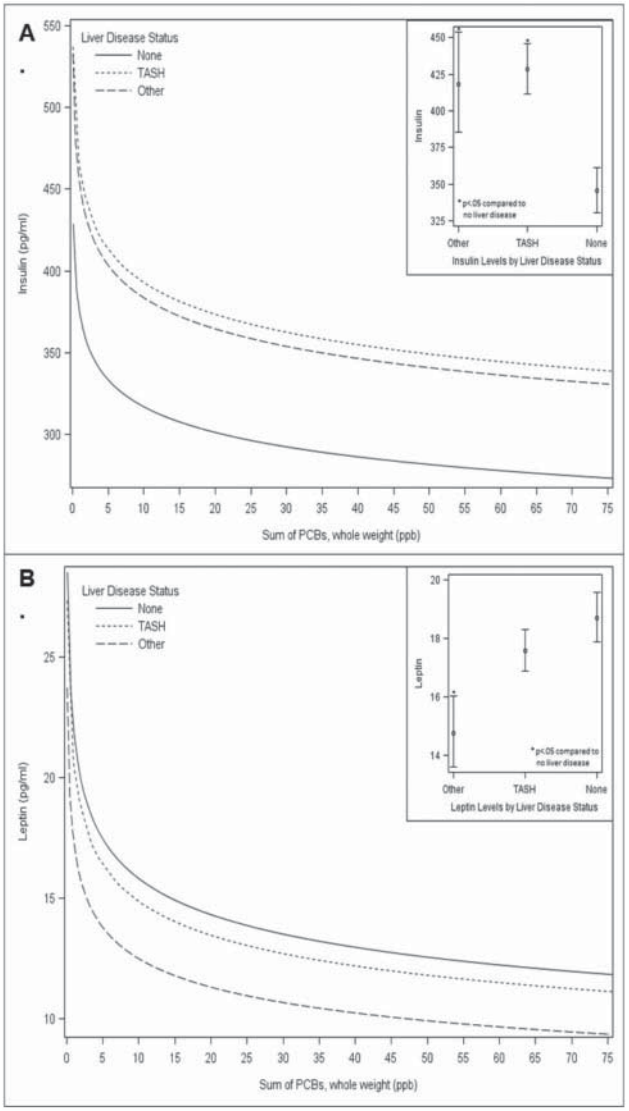

Figure 1. Insulin and leptin levels decrease with increasing ΣPCB serum levels. Regression curves and insets with means and standard errors are shown. Across all categories and in the entire ACHS population, insulin (A) and leptin (B) decreased significantly with increasing total PCB load. Both TASH and Other liver disease categories had significantly elevated means for insulin compared with individuals without biomarker-indicated liver disease (None) (A, inset). Means for leptin were significantly lower in the Other liver disease category, compared with no liver disease (None) (B, inset).

studies will be published separately in the future providing further characterization of the liver disease in this cohort. Because fatty liver disease is a widespread and serious health problem that may arise through very different mechanisms, increasingly thorough characterization of the effects of PCBs in Anniston will improve the understanding of liver disease epidemiology and pathogenesis.

Ten PCB congeners (PCBs 28, 44, 49, 52, 66, 101, 110, 128, 149, and 151) were positively associated with both TASH and the CK18 M65 liver necrosis biomarker (Table 2). An additional 5 PCBs (105, 172, 178, 187, and 195) were significantly associated with CK18 M65, but not TASH. Some of these PCBs (66, 105, 151, 172, 178, and 187) have previously been associated with increased ALT in other cohorts (Cave *et al.*, 2010a; Kumar *et al.*, 2014a, 2014b; Yorita Christensen *et al.*, 2013). One of the PCB congeners (PCB 105) associated with TASH and/or CK18 M65 elevation has dioxin-like activity, while others have estrogenic (44, 49, 52, 101, and 110) or phenobarbital-like effects (44, 49, 52, 101, 128, 149, 151, 187, an 195) (Warner *et al.*, 2012). Perhaps activation of the AhR, estrogen receptor, or the *CAR* was involved in PCB-related TASH. Although 4 congeners were associated with increased CK18 M30, an inverse association was observed between ΣPCBs and this apoptosis biomarker (Supplementary Table 1). Notably, activation of the PCB receptor *Car*, was found to be antiapoptotic in a mouse model of cholestatic liver disease (Beilke *et al.*, 2009). Although not directly tested in this study, it is conceivable that PCBs could mediate a transition from NASH to TASH by decreasing apoptosis and thereby promoting more proinflammatory necrotic hepatocyte death. Indeed, PCBs were a "second hit" in the transition from diet-induced steatosis to more advanced liver disease in animal studies (Hennig *et al.*, 2005; Shi *et al.*, 2012; Wahlang *et al.*, 2014b, 2016). In ACHS-II, additional dioxins including nonortho PCBs were measured (Birnbaum *et al.*, 2016). The additive impact of dioxin-like chemicals on liver disease, summarized by the total dioxin toxic equivalency (Van den Berg *et al.*, 2006) is currently being analyzed. This ongoing study will help to further elucidate the potential role of AhR activation in environmental TASH.

In ACHS, the prevalence of liver disease differed by demographic variables such as sex and race/ethnicity. Liver disease affected males (67.2%) and nonHispanic whites (70.2%) disproportionately. The sex difference is consistent with a recently published report documenting increased mortality from hepatic disease in PCB-exposed men from the Yucheng and Yusho cohorts (Li *et al.*, 2015). Men have increased susceptibility to NAFLD (Pan and Fallon, 2014), however observed gender differences might also be explained by sex-specific effects of estrogenic PCB congeners. Genetic polymorphisms may contribute to racial/ethnic differences in NASH susceptibility (Anstee and Day, 2013; Houghton-Rahrig *et al.*, 2014; Pan and Fallon, 2014). For example, ethnic differences in the distribution of null alleles in the NASH susceptibility gene (patatin-like phospholipase domain containing protein 3 [PNPLA3]), have been reported (Pan and Fallon, 2014). We postulate that gene-environment interactions may also influence TASH; and that these interactions may have contributed to the ethnic differences observed in this study. In addition to PNPLA3, candidate genes include the pregnane X receptor (*PXR*), which is a PCB receptor (Wahlang *et al.*, 2014a) with at least 2 polymorphisms previously associated with NAFLD severity (Sookoian *et al.*, 2010).

Although steatohepatitis has been associated with cytokine abnormalities (Joshi-Barve *et al.*, 2015; Wahlang *et al.*, 2013a), relatively less is known about the effects of PCBs on these biomarkers. In a cohort of 992 elderly Swedish subjects, ΣPCBs was significantly associated with vascular cell adhesion protein 1 but not IL-6, MCP-1, or TNFα (Kumar *et al.*, 2014b). In mice co-exposed to high fat diet, Aroclor 1260 increased TNFα, IL-6, and PAI-1 (Wahlang *et al.*, 2014b, 2016). Adiponectin was inversely associated with PCB 28, 138, and 153 in a study of 98 Koreans (Lim and Jee, 2015); but ΣPCBs was not associated with adiponectin levels in the Great Lakes Sport Caught Fish Consumers Study ($n = 413$) (Turyk *et al.*, 2015). Leptin receptor expression was decreased in PCB-exposed children (Ghosh *et al.*, 2013); and PCB-exposures induced leptin resistance in the 3T3-L1 cell culture model while increasing both TNFα and IL-6 production (Ferrante *et al.*, 2014); In this study, ΣPCBs was associated with increased TNFα and IL-6 in the model which adjusted for lipids only (Table 4). Although ΣPCBs was associated with increased

Table 6. Summary of Significant Effects of Liver Disease Categories and ΣPCBs on Cell Death Mechanisms and Serologic Biomarkers of Systemic Inflammation and Intermediary Metabolism

	Predominant Effect On Hepatocyte Death Mechanisms	PCB Congeners Associated With Either The Liver Disease Category or The Predominant Cell Death Mechanism	Associated Adipocytokines	Glucose Metabolism	Lipid Metabolism
TASH	↑ Necrosis	28, 44, 49, 52, 66, 101, 105, 110, 128, 149, 151, 172, 178, 187, 195[b]	↑ IL-1β[c], IL-6[c], PAI-1[c, d], MCP-1[c]	↑ HOMA-IR[c], HOMA-B[c], Insulin[c]	↑Triglycerides[d]
Other	↑ Apoptosis	44, 49, 52, 110[b]	↑ IL-6[c], PAI-1[d] ↓ Leptin[c]	↑ HOMA-IR[c, d], Insulin[c, d]	↑Triglycerides[d]
ΣPCBs	↓ Apoptosis[a]		↑ TNFα[e], IL-6[e], Adiponectin[e] ↓ Leptin[c]	↓ HOMA-IR[c], HOMA-B[e], Insulin[c, f]	[f]

[a]Supplementary Table 1.
[b]Supplementary Table 2.
[c]Supplementary Table 5.
[d]Supplementary Table 3.
[e]Supplementary Table 4.
[f]The possible effects of PCBs on triglycerides were not evaluated given the lipid adjustment procedures used in Tables 4 and 5.

adiponectin in the model that adjusted for lipids only (Table 4), ΣPCBs was associated with decreased leptin in the fully adjusted model (Table 5). Importantly, in the fully adjusted model, 26 PCB congeners were associated with decreased leptin. If PCBs decrease leptin expression while simultaneously inducing leptin resistance, this could be an important potential mechanism for PCB-related metabolic dysfunction. Additional serum adipocytokines have been measured in ACHS-II, and possible associations with PCB and dioxin exposures will be reported in the future.

A high prevalence of diabetes (27%) has previously been reported in the ACHS (Silverstone *et al.*, 2012), and the impact of PCBs on cardiovascular disease and its risk factors, including diabetes, has recently been reviewed (Perkins *et al.*, 2016). In this study, ΣPCBs was significantly associated with decreased HOMA-B in the model which adjusted for lipids only (Table 4). There was a trend ($p = .08$) towards decreased HOMA-B in the fully adjusted model (Table 5). ΣPCBs and 9 individual high molecular weight congeners were associated with decreased HOMA-IR in the adjusted model. ΣPCBs was inversely associated with insulin in both models. Adjustments were not made for diabetes medications, so it is difficult to draw firm conclusions from these data. Nonetheless, these data are generally consistent with the findings from our recently published mouse model. In that experiment, C57Bl/6 male mice were fed a 42% milk fat diet for 12 weeks with or without coexposure to a high molecular weight PCB mixture (Aroclor 1260) at a dose designed to model ACHS (Wahlang *et al.*, 2016). PCB treatment induced steatohepatitis, decreased HOMA-B and HOMA-IR but did not change glucose tolerance. Hepatic gluconeogenesis, glucose transporters, physical activity, food intake, and respiratory exchange rate appeared to be regulated, in part, by interactions between PCBs and the nuclear receptors *Pxr* and *Car* (Wahlang *et al.*, 2016). These studies clearly demonstrate significant derangements in glucose metabolism occurring in the context of PCB-related liver disease. The mechanisms underpinning the complicated effects of PCBs on intermediary metabolism in steatohepatitis require further elucidation. However, based on the results of the present study, pancreatic β cell dysfunction, rather than insulin resistance, may be involved in the diabetes that is associated with some PCB exposures.

In conclusion, the 60.2% prevalence of liver disease in the PCB-exposed ACHS population is among the highest ever reported for a residential cohort. Liver disease was most often characterized by hepatocellular necrosis associated with proinflammatory cytokine elevation and insulin resistance consistent with TASH. Exposures to 10 individual PCB congeners were associated with TASH. We suggest that there should be a greater focus on exposures to endocrine and metabolism disrupting chemicals in hepatology practice.

HIGHLIGHTS

- The Anniston Community Health Survey is a PCB-exposed residential cohort previously found to have a high prevalence of overweight/obesity and diabetes. This study determined a high prevalence of biomarker indicated liver disease (60.2%) associated with sex and race/ethnicity differences.
- Most of the liver disease cases (80.7%) were categorized as TASH, which was characterized by predominant hepatocellular necrosis.
- TASH was associated with increased exposures to ortho-substituted PCB congeners, dyslipidemia, insulin resistance, and proinflammatory cytokine elevations consistent with PCB-related steatohepatitis.
- Σortho-substituted PCBs was associated with decreased pancreatic insulin production, insulin resistance, leptin, and hepatocyte apoptosis, while several proinflammatory cytokines and adiponectin were increased. The diabetes previously associated with PCB exposures may be related to the combination of decreased pancreatic insulin production and increased insulin resistance due to PCB-related TASH.
- This is possibly the largest environmental liver disease study performed investigating mechanistic biomarkers and the most comprehensive analysis of PCBs and adipocytokines. It provides insight into the mechanisms of PCB-related endocrine and metabolic disruption in liver disease and diabetes.

SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

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