SENSITIVE AND SPECIFIC LIVER INJURY BIOMARKERS: ELEVATED LIVER DISEASE IN ORGANOCHLORINE TOXICANT EXPOSED COHORT

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Introduction

Fatty liver disease is a spectrum ranging from fat accumulation in the liver (simple steatosis) to fat accumulation with inflammatory cell infiltration (steatohepatitis) to cirrhosis (scarring of the liver). Liver failure or cancer may eventually result from chronic inflammation. Steatohepatitis is histologically similar whether the disease is secondary to alcohol abuse (alcoholic steatohepatitis, or ASH), obesity (nonalcoholic steatohepatitis, or NASH), or exposure to chemicals (toxicant-associated steatohepatitis, or TASH). The cellular processes leading to liver damage, however, may be distinct for each case, reflected by differences in the levels of specific protein biomarkers released into the blood upon hepatocyte damage and death.

Two groups of biomarkers used clinically to diagnose liver injury are the transaminases alanine aminotransferase (ALT, also known as SPGT) and aspartate aminotransferase (AST, or SGOT), and recently Cytokeratin 18 (CK18), a structural protein found in liver cells. Both may be considered "spillage proteins", in that they are intracellular components released into the blood upon hepatocyte death. CK18 detection, however, is accomplished by antibody capture (Enzyme-Linked Immunosorbent Assay, or ELISA), which quantifies the amount of the protein in the serum, while transaminase levels are inferred from the enzyme activity of the protein in blood samples.

In 2007, our laboratory reported a distinct form of steatohepatitis in a group of non-obese plastics industry workers highly exposed to vinyl chloride, which we have classified as toxicant-associated steatohepatitis (TASH). Although exposed workers had a very high prevalence of biopsy-proven NASH, ALT and AST in this population were normal.[1] Importantly, because current US standards require the monitoring agency responsible for worker safety to employ ALT and AST to assess liver injury in industrial chemical workers, cases of TASH in some industrial settings may go undetected until late in the disease progression.

In subsequent work, we therefore used ELISA evaluation of CK18 forms as a measure of liver injury in chemical-exposed individuals. CK18 is a component of the cytoskeleton of cells of epithelioid origin, such as skin, lungs and liver. Because the liver is the largest internal organ composed of epithelioid cells and CK18 is abundant in hepatocytes serum CK18 is a relatively specific and highly sensitive biomarker of hepatocyte injury.[2] In addition to the absolute level of CK18 in the blood, detectable by the CK18 M65 assay, we are able to assess the levels of the caspase-cleaved fragment CK18-M30. This allows us to determine the levels of hepatocyte death attributable to apoptotic (programmed, regulated cell death) vs. necrotic (disorganized and unregulated cell death) mechanisms. Apoptosis appears to be the primary mechanism of cell death in NASH. Previous publications have shown that CK18 M30 fragment reading of >217 U/L is associated with a 95% probability of having NASH in the absence of other known sources of liver disease.[3] In TASH, however, elevations in CK18-M65 are accompanied by normal CK18-M30 (<200 U/L), suggesting a primarily necrotic mechanism of hepatocyte death. We have identified several populations exposed to organochlorine compounds that display this pattern as well, including residents of a neighborhood surrounding the site of the former Black Leaf organochlorine pesticide manufacturing plant in Louisville, KY (Black Leaf), and a large cohort of individuals residing in Anniston, Alabama, who were exposed to polychlorinated biphenyl (PCB)-containing waste released by a former Monsanto PCB manufacturing facility.

Previous studies involving the Anniston cohort have described increased prevalence of hypertension (high blood pressure) corresponding to PCB exposure level, and increased prevalence of diabetes[4-5], however, liver disease has not been previously evaluated in this population. Here we present measured serum levels of the liver injury biomarkers CK18 M56 and M30 from the original ACHS participants and also from a subset of the Anniston cohort who were resampled in 2014 (ACHS II).

Materials and Methods

Collection of samples and analysis of PCB concentrations

The ACHS samples examined in the course of this study were collected in 2005-2007.[4-9] Briefly, in the two-stage sampling procedure, 3,320 households were randomly chosen from a list of all residences within the city limits. Residences in West Anniston, the site of the former PCB manufacturing facility, were oversampled. Contacts were made with 1,823 of the targeted households. Of these, 1,110 households consented to participate, and one adult (>18 years of age) from each consenting household was selected to complete the survey, resulting in an overall participation rate of 61% (1,110/1,823); 774 of those volunteered to provide blood sample. Participants who completed both the survey and a clinic visit, had available leftover serum sample, as well as biometric and questionnaire response data were included in this analyses. Serum biomarkers of liver injury were analyzed in our laboratory in 2013 for 738 samples from ACHS.

PCB concentrations were measured at the National Center for Environmental Health Laboratory of the Centers for Disease Control and Prevention (Atlanta, GA) as previously described.[7]

In 2014, 352 members of the ACHS cohort were re-sampled in order to provide time-course data and to evaluate serum levels of dioxins, dibenzofurans and non-ortho dioxin-like PCBs not analyzed in ACHS. We once again analyzed CK-18 levels in this subset (ACHS II), as well as clinical biomarkers ALT, AST, Alkaline Phosphatase (ALP), and direct/total bilirubin.

Analysis of serum liver injury biomarkers

The serum liver injury biomarker CK18 was assayed in our laboratory using M65 (PEVIVA 10020) and M30 (PEVIVA 10010) ELISA kits. Elevations beyond the normal cutoff of 300 U/L for M65 were considered an indication of increased hepatocyte death, while elevations in M30 above the cutoff of 200 U/L were considered evidence of specifically apoptotic hepatocyte death. Clinical biomarkers of liver injury (ALT, AST, ALP, direct/total bilirubin) were evaluated in the clinical laboratory of University Hospital, Louisville, KY.

Statistical Analysis

ACHS and ACHS II participants were stratified into three groups based on serum biomarker-indicated liver injury: no liver disease (None, M30<200 U/L and M65<300 U/L), toxicant-associated steatohepatitis (TASH, M30<200 U/L and M65>300 U/L), and other liver disease (Other, M30>200 U/L).

Counts and percentages were calculated for the main predictors for the ACHS population and by liver disease status. Differences in log-transformed means and frequencies by liver disease status were tested with a one-way ANOVA or chi-square test, respectively. Individual injury, inflammation, or metabolic function biomarkers were analyzed with multivariable generalized linear models to assess the relationship between serum PCB levels and each outcome. Biomarkers, PCB levels, and lipid levels were log-transformed. Unless noted elsewhere, all analyses were adjusted for liver disease status, age, body mass index (BMI, kg/m^2), gender, race (African American/Black vs. White), diabetes status, total lipid levels and alcohol use. Regression lines were plotted for each category of liver disease status for all participants. Statistical analyses were performed with SAS version 9.4 and used a statistical significance level of 0.05.

Results and Discussion

Table 1. Demographic characteristics of ACHS participants by liver disease status.

Characteristic	Liver disease status			Total
	None (n = 294)	TASH (n = 359)	Other (n = 85)	(n = 738)
Age (years)[a]	54.06±15.68	55.99±16.25	51.46±15.06	54.70±15.93
BMI (kg/m^2)	31.47±7.78	30.87±7.62	32.09±7.68	31.25±7.69
∑PCBs (ng/g whole weight)	6.37±9.11	7.22±14.43	5.41±10.28	6.67±12.11
Total lipids (mg/dL)[a]	611.11±131.71	643.62±163.61	656.91±192.35	632.20±156.30
Gender[a]				
Male	72 (33%)	123 (56%)	26 (12%)	221
Female	222 (43%)	236 (46%)	59 (11%)	517
Race/ethnicity[a]				
Non-Hispanic White	117 (30%)	223 (57%)	53 (13%)	393
African-American/Black	177 (51%)	136 (39%)	32 (9%)	345 (46.75)

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

[a] p<=0.05 in one-way ANOVA (means) or Pearson chi-square test, across liver disease categories.

Participant characteristics are summarized in Table 1. In general, the participants were more likely to be female, ≥50 years old and obese (BMI ≥30 kg/m2). Non-Hispanic White individuals (57%) and males (56%) within this population were most likely to be categorized as having TASH, based on CK18-M65 and CK 18-M30 levels. African Americans (39%) and females (46%) had lower prevalence of TASH; both of those differences were statistically significant.

Table 2: Prevalence of liver disease in ACHS and ACHS II samples based on CK18 level and pattern.

Population	CK-18 Indicated liver disease status			
	None	TASH	Other	Total
ACHS	294 (40%)	359 (49%)	85 (12%)	738
ACHS II	131 (37%)	163 (46%)	58 (16%)	352
Abnormally High ALT[a]	*3/131*	*15/163*	*21/58*	*39/352 (11%)*

[a] ACHS II samples only.

The prevalence of biomarker-indicated liver disease based on CK18 level in ACHS and ACHS II populations is shown in Table 2. Prevalence of TASH plus Other liver disease was over 60% for both ACHS and ACHS II compared to the estimated global and North American prevalence of liver disease of 25.2% and 24.3%, respectively.[10] ALT was evaluated only in ACHS II. The overall prevalence of abnormal ALT in ACHS2 (11%) approximated closely that of the general US population with unexplained ALT elevation (10.6%).[11]

In this study we used serum levels of CK18-M65 and CK18-M30 to categorize ACHS and ACHS II participants by liver disease category. Based on this sensitive biomarker, we report a 60% prevalence of liver disease in the ACHS and ACHS II, which approaches 65% in males and 70% in self-identified Non-Hispanic Whites. Despite this extraordinarily high prevalence, our CLIA lab reported abnormally high liver enzymes in 11% of the ACHS II samples – a prevalence similar to that of the general US population. Comparison between ACHS and ACHS showed no significant difference in the population mean for liver injury biomarkers CK18-M65 or CK18-M30. The proportion of the population assigned to the TASH liver category based on CK18-M65 and CK18-M30 patterns was also constant. The criteria used to assign liver disease category was based on our previous definitions from plastics and elastomer workers, however subsequent research NASH cohorts suggest that a NASH patient with a CK-18 M30 fragment reading of 217 U/L corresponds approximately to a CK-18 M65 reading of 260 U/L

(unpublished data). Thus our cutoffs for TASH designation at CK-18 M65>300 U/L and a CK-18 M30<200 U/L are conservative and it is possible that some patients in the "no liver disease" category have borderline liver disease and some in the "other liver diseases" category with a very high CK-18 M65 and a CK-18 M30>200 U/L may have TASH. Further research is needed to establish whether a similar mechanism underlies liver injury observed in populations exposed to organochlorine contaminants in their environments.

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