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Research Article

Environmental chemicals and endogenous metabolites in bile of USA and Norway patients with primary sclerosing cholangitis

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Abstract

Primary sclerosing cholangitis (PSC) is a complex bile duct disorder. Its etiology is incompletely understood, but environmental chemicals likely contribute to risk. Patients with PSC have an altered bile metabolome, which may be influenced by environmental chemicals. This novel study utilized state-of-the-art high-resolution mass spectrometry (HRMS) with bile samples to provide the first characterization of environmental chemicals and metabolomics (collectively, the exposome) in PSC patients located in the United States of America (USA) ($n = 24$) and Norway ($n = 30$). First, environmental chemical- and metabolome-wide association studies were conducted to assess geographic-based similarities and differences in the bile of PSC patients. Nine environmental chemicals (false discovery rate, FDR < 0.20) and 3143 metabolic features (FDR < 0.05) differed by site. Next, pathway analysis was performed to identify metabolomic pathways that were similarly and differentially enriched by the site. Fifteen pathways were differentially enriched ($P < .05$) in the categories of amino acid, glycan, carbohydrate, energy, and vitamin/cofactor metabolism. Finally, chemicals and pathways were integrated to derive exposure–effect correlation networks by site. These networks demonstrate the shared and differential chemical–metabolome associations by site and highlight important pathways that are likely relevant to PSC. The USA patients demonstrated higher environmental chemical bile content and increased associations between chemicals and metabolic pathways than those in Norway. Polychlorinated biphenyl (PCB)-118 and PCB-101 were identified as chemicals of interest for additional investigation in PSC given broad associations with metabolomic pathways in both the USA and Norway patients. Associated pathways include glycan degradation pathways, which play a key role in microbiome regulation and thus may be implicated in PSC pathophysiology.

Keywords: bile; exposome; machine learning; multi-omics; primary sclerosing cholangitis

Introduction

Primary sclerosing cholangitis (PSC) is a rare, chronic cholestatic liver disease characterized by inflammation and fibrosis of the bile ducts and impaired bile flow that leads to end-stage liver disease and hepatobiliary neoplasia.[1] Liver transplantation is currently the only evidence-based option for advanced disease—no drug therapy exists to improve transplant-free survival.[2] PSC likely develops from a combination of genetic and environmental contributors, but these are incompletely understood, either individually or together.[3-5] These complex interactions between environment and host have galvanized research into the exposome in PSC.[6]

The exposome is defined as the cumulative environmental influences and corresponding biological responses throughout the lifespan.[7] While endogenous processes can be characterized using well-developed -omic technologies (eg, genomic, proteomic, transcriptomic, and metabolomic instruments), the ability to characterize environmental exposures on the -omic scale has been limited by challenges in measuring complex exposure profiles that potentially include thousands of exposure biomarkers.[7-10] However, recent advances in high-resolution mass spectrometry (HMRS) for small molecule profiling facilitate improved, -omic-scale investigation of the exposome.[11] This enables measurement and analysis of

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internal chemical doses and biological responses, with sufficient exposome coverage to investigate the complex relationships between potential disease drivers, biological effects, and clinical outcomes.

Through integrative analysis of HRMS-detected exposures and endogenous metabolic pathways, a relationship between chemical exposure and biological response has been identified in the plasma of patients diagnosed with PSC.[6] This suggests a critical role for environmental exposures in PSC pathophysiology. Given that PSC is a disease of the bile ducts, characterizing the exposome of bile, which directly contacts the diseased tissue, is imperative for advancing our molecular understanding of the disease. It is well known that the excretion of biotransformed chemicals (such as via glucuronidation and sulfation) into bile is a major metabolic elimination mechanism. Parent chemicals as well as conjugated metabolites may enter bile,[12] yet in PSC, these parent compounds and metabolic conjugates have not been identified. The only human PSC bile metabolomic study to date suggested aberrant bile formation in PSC ($n = 7$), compared to individuals with noncholestatic end-stage liver disease ($n = 19$), and nondisease controls ($n = 12$).[13] However, those specimens were collected as part of a liver transplant procedure. Additional characterization of bile exposures from samples collected via endoscopic retrograde cholangiopancreatography (ERCP), a more representative procedure for bile collection, is warranted for better biological understanding of PSC.

In this work, we utilized a novel HRMS-based strategy to characterize environmental chemicals and endogenous metabolites present in the bile of patients with PSC, providing the first comprehensive exposome characterization of bile in complex liver disease (Figure 1). We hypothesized that integrative network analysis between different geographical locations would (1) provide insights into the shared and distinct bile exposures of patients with PSC and (2) facilitate exploration of the role of environmental chemicals in any observed differences. The statistical interactions between environmental chemicals and endogenous metabolites derived from network analysis may inform potential mechanisms underlying the pathophysiology of PSC.

Methods

Study design and population

The sample comprised of 54 patients with PSC ($n = 24$ who received care at Mayo Clinic in Minnesota, USA, and $n = 30$ who received care at Oslo University Hospital in Oslo, Norway) (Table 1). As collecting bile from individuals without liver disease is challenged by the invasiveness and risk of complications of ERCP, this cohort included only patients with PSC aimed at bile characterization. All patients met the diagnostic criteria for PSC according to the guidelines published by the American Association for the Study of Liver Diseases and the European Association for the Study of the Liver: (1) biochemical evidence of chronic cholestasis (≥6 months); (2) cholangiographic findings of multifocal strictures alternating with segmental dilatations in the bile ducts and/or histological findings consist with PSC; and (3) causes of secondary sclerosing cholangitis have been excluded.[14,15] Medical charts of all patients were reviewed for the accuracy of PSC diagnosis and related clinical complications. For each patient, the following data were extracted: sex, age at the time of diagnosis of PSC, date of last known clinical follow-up, liver biochemistry measurement performed within 3 months of bile collection (alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, and total bilirubin), inflammatory bowel disease (IBD) status, progression to/development of clinically important endpoints (eg, compensated and decompensated cirrhosis, cholangiocarcinoma, liver transplantation, and development of colorectal cancer), and medications recorded at the time of bile collection (Table 1). Bile was collected during prescheduled ERCP as part of the patient's clinical care. Collected specimens were kept and transported on ice, centrifuged to remove debris, and aliquots were stored frozen at −80°C until use. Research procedures were conducted in accordance with the approval of the Institutional Review Board at the Mayo Clinic and the Research Ethics Committee at Oslo University Hospital. Written informed consent was obtained from all participants.

High-resolution exposomics

Environmental chemicals were measured in bile using gas chromatography high-resolution mass spectrometry (GC–HRMS) and liquid-chromatography high-resolution mass spectrometry (LC–HRMS). GC–HRMS was utilized as the primary environmental chemical platform as many environmental chemicals are hydrophobic, semi-volatile, and present ionization challenges with popular LC–HRMS methods.[11] LC–HRMS data (described in the "High-resolution metabolomics" section) were used as an additional data source for annotating environmental chemicals.[16] The analytes selected for targeted GC–HRMS analysis were based on a library of organic environmental chemicals that are widely used and occur frequently in the environment. This includes the common persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers, and pesticides. These bioaccumulate over time, disrupt metabolic and endocrine function, and have a high toxicity.[17]

In addition to the routinely biomonitored chemicals (eg, by the National Health and Nutrition Examination Survey program), we also included contemporary contaminants such as polycyclic aromatic hydrocarbons (PAHs), insecticides/pesticides, flame retardants, plasticizers, flavoring agents and food additives, phthalates, and chemicals used for personal care. It is hypothesized that these may be present in bile as bile provides a major route for metabolic elimination of conjugated chemicals (eg, through glucuronidation or sulfation) and parent compounds.[12] These chemicals may also be subject to enterohepatic circulation mediated by the bile, increasing their retention time (RT) within the bile ducts, liver, blood, and digestive system.

Briefly, for GC–HRMS sample profiling, 13C-labeled chemical standards, each with 99% isotope enrichment, were spiked at a final concentration of 1 ng/mL for quality control and assurance, as previously reported.[11,18] Environmental chemicals in 150 μL bile samples were extracted with 50 μL formic acid followed by 200 μL hexane–ethyl acetate (2:1 v/v, ≥99% pure, Sigma-Aldrich). The chilled mixture was shaken vigorously and centrifuged to obtain the organic supernatant, which was further cleaned with high-purity $MgSO_4$. $MgSO_4$ provides similar efficacy and similarly high reproducibility for cleaning compared with dispersive solid phase extraction.[11] The bile extracts were analyzed with three injections using GC–HRMS with a Thermo Scientific Q Exactive GC hybrid quadrupole Orbitrap mass spectrometer with 2 μL per injection. Data were collected from 3 to 24.37 min with positive electron ionization mode (+70 eV), scanning from m/z 85.0000 to 850.0000 with a resolution of 60 000. National Institute of Standards & Technology Standard Reference Materials (SRM) 1958 and SRM-1957 were analyzed in every batch of 20 samples to support quality control and batch effect evaluation. Contamination and carryover were assessed in isooctane washes, solvent blanks, and method blanks, which were run at the

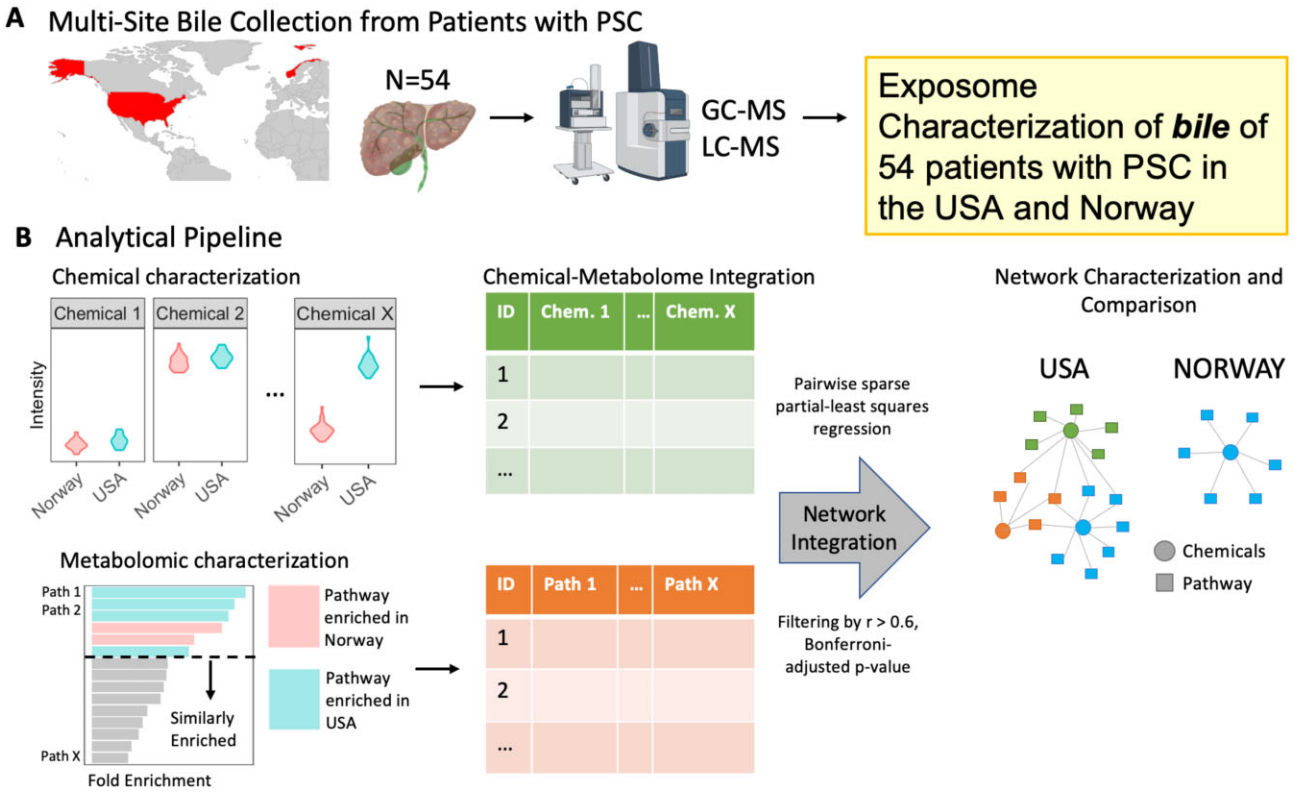

Figure 1. Conceptual overview. (A) Bile samples were collected from patients with PSC located in the USA and Norway. Samples were assayed for environmental chemicals and metabolites using GC and LC–HRMS. (B) Analytical pipeline. Intensities of 92 chemicals were characterized and compared across sites. Metabolomic pathway analysis was performed, and pathways were compared for enrichment by the site. All identified chemicals and pathways were integrated using a network science approach to derive chemical–metabolite association networks that best characterize each site. Site-specific analyses were done given observed differences in chemical intensities and metabolomic pathway enrichment by site. Figure created with the help of Biorender.com.

beginning of each batch by monitoring of peak baseline. Raw data were extracted using XCMS.[19] Ninety-two environmental chemicals met the criteria for "Level 1" identification[11] by comparison of accurate mass, fragmentation patterns, and RT to an in-house library of authentic standards run on the same instrument using identical analytical parameters.[11] Average peak intensities of the three technical replicates per sample were used to quantitatively represent levels of environmental chemicals.

High-resolution metabolomics

Untargeted, LC–HRMS profiling of bile was completed in batches of 40 study samples using established methods with two platforms, C_{18} chromatography with negative electrospray ionization (ESI), and hydrophilic interaction liquid chromatography (HILIC) with positive ESI, as described in detail.[20] Briefly, 65 µL bile aliquots were treated with two volumes of ice-cold acetonitrile to precipitate the proteins. A mixture of 10 stable isotope internal standards was included for quality control as previously reported. Following 30 min incubation on ice, samples were centrifuged for 10 min at 16 100*g* at 4 °C. The supernatants were analyzed with dual chromatography-coupled HRMS (Thermo Scientific HF Q-Exactive). The HRMS was operated in full scan mode at 120 000 resolution and mass-to-charge ratio (*m*/*z*) range of 85–1275. Raw data files were extracted and aligned using the R package apLCMS[21] with modifications by xMSanalyzer (for details, see Supplementary materials).[22] Amongst additional functions, xMSanalyzer evaluates the quality of each feature and removes the low-quality features.[22] For example, features with <75% correlation amongst the three technical replicates were deemed low quality and removed. Uniquely detected peaks consisted of *m*/*z*, RT, and ion abundance referred to as metabolite features. Peak extraction detected 9735 C18 and 1522 HILIC metabolite features. For quality control purposes, a 10% feature missingness threshold was employed, leaving 3526 C18 features and 5978 HILIC features for inclusion in subsequent analyses. Peak annotation for endogenous metabolites was performed following metabolome-wide association study (MWAS) (described under the "Statistical analysis" section) using the mummichog 2.0 algorithm[23] on Metaboanalyst[24] and the Homo sapiens MFN pathway library, a manually curated library that originates from numerous sources including KEGG, BiGG, and Edinburgh Model.[24] Peak annotation for environmental compounds in LC–HRMS data was conducted using xMSannotator[16] with the Human Metabolome Database (HMDB) (for details, see Supplementary materials).[25] xMSAnnotator uses a multi-stage clustering algorithm to derive compound annotation and confidence scores, which range from 0 (no confidence) to 3 (high confidence).[16] Chemical annotations derived from xMSAnnotator with high or medium confidence scores (≥2) and with the M + H adduct (positive mode) or M−H (negative mode) are equivalent to the Level 2 confidence score by the Mass Spectrometry Imaging (MSI) criteria.[26] Lower confidence annotations (MSI Level 4) were derived from HMDB and the Metlin mass spectrometry databases at 5 p.p.m. tolerance.

Statistical analysis

All statistical analyses were implemented in R version 4.0.3[27] using RStudio version 1.3.[28]

Table 1. Demographics and clinical characteristics

	USA	Norway
Patients (N)	24	30
Sex (N male, N Female)	13M, 11F	15M, 15F
Age at PSC Diagnosis [Median (Min, Max)]	40 (11, 73)	37 (15, 64)
Age at Bile Collection [Median (Min, Max)]	50 (20, 77)*	43 (17, 70)*
Duration of PSC (years) [Median (Min, Max)]	7.6 (0.3, 38.1)	4.3 (0.1, 21.8)
Laboratory tests		
Total bilirubin (mg/dL) [Median (Min, Max)]	1.5 (0.4, 13.6)	0.76 (0.23, 12.3)
Alkaline phosphatase (IU) [Median (Min, Max)]	322 (96, 637)	244 (36, 749)
AST (IU) [Median (Min, Max)]	70.5 (16, 157)	48 (16, 395)
ALT (ID) [Median (Min, Max)]	82 (17, 362)	66 (11, 521)
Comorbidities		
Inflammatory Bowel Disease (% yes)	66%	75%
Colorectal Cancer (% yes)	12.5%	0%
Cirrhosis (% yes)	4.2%	3.3%
Splenomegaly (% yes)	4.2%	3.3%
Varices (% yes)	4.2%	3.3%
Ascites (% yes)	4.2%	0%
Cholangiocarcinoma (% yes)	0%	0%
Prior liver transplant (% yes)	0%	0%
Pharmacotherapy		
Vitamins/Supplements (%)	62%**	20%**
Blood Pressure Medications (%)	38%*	10%*
Ursodiol (%)	29%	33%
Gastrointestinal Medications (%)	29%	20%
Endocrine Medications (%)	25%	10%
Antidepressant (%)	25%**	0%**
Inflammatory Bowel Disease (IBD) Medications (%)	46%	47%
IBD: Mesalamine (Asacol), Mesalamine (Asacol) +Infliximab, or Mesalamine (Asacol) +Vedolizumab (N)	6	10
IBD: Balsalazide (N)	1	2
IBD: Infliximab (N)	0	1
IBD: Sulfasalazine (N)	1	1
IBD: Budesonide (N)	1	0
IBD: Mercaptopurine (N)	2	0

* $P < .05$
** $P < .01$ significantly different according to Wilcoxon rank sum test or Fischer's exact test. Laboratory test data are based on available measures taken within 3 months of bile collection from 11 US patients and 29 Norway patients. IBD rates reflect patients with either Crohn's disease or ulcerative colitis compared to those without either condition. Pharmacotherapy prevalence rates are reported for drugs prescribed to ≥20% of the patients at either geographical location, with drug names listed for IBD medications.

Exposomic analysis - Environmental-wide association study (EWAS)

GC–HRMS and LC–HRMS assayed exposures were analyzed separately because the GC–HRMS workflow produced 92 confidently identified environmental compounds, while the LC–HRMS workflow produced annotations for environmental compounds. Peak intensities were log2 transformed and standardized using their median and interquartile range prior to all statistical analyses. Following transformation and standardization, hierarchical clustering using Euclidean distance and complete linkage was performed on both patients and GC–HRMS identified chemicals, by site, to assess whether groups of patients with similar clinical and demographic features would cluster by bile chemical profiles. Next, multiple linear regression was used to evaluate the association of environmental chemicals with geographical location (EWAS). In this EWAS, for each chemical, the log2-transformed intensity was modeled as a function of location (USA or Norway), controlling for age, sex, and duration of PSC, which are known to influence biochemical concentrations and/or disposition.[29-31] To reduce the number of false positives, all chemicals associated at FDR <0.20 with the location were considered significant. Additionally, given the high comorbidity of IBD with PSC,[32] a second, exploratory analysis was conducted to assess potential associations (FDR < 0.20)[6] of GC–HRMS-identified environmental chemicals with IBD status. This analysis controlled for patients' location, sex, age, and duration of PSC. LC–HRMS-annotated environmental exposures were manually curated based on accurate mass matches to dietary, environmental chemical, and microbiome metabolites from xMSannotator.[16]

Metabolome-wide association study

MWAS was performed to identify site-associated metabolic features (reported by *m/z* and RT).[33] Data pre-processing and analyses were performed separately for the C18 and HILIC columns.[6,34] Multiple linear regression was utilized to model the log2 feature intensity as a function of site (USA or Norway), controlling for age, sex, and duration of PSC (as in EWAS). Due to the large number of features, an FDR threshold of <0.05 was used to account for multiple testing and to reduce false positives. This more stringent LC-data FDR threshold compared with the GC threshold (FDR < 0.20) was implemented to further reduce the possibility of false positives in the LC analyses, which were based on the untargeted chemical intensities, compared to the GC analyses based on identified chemicals.

Metabolomic pathway analysis

Pathway analysis was performed using Mummichog[23] implemented through MetaboAnalyst.[24] Mummichog enables the identification of pathways enriched by a condition (presently, geographical location) from untargeted metabolomics data without a priori identification of metabolites. Mummichog predicts metabolite identity and calculates pathway enrichment using Fisher's exact test.[23] A list of all detected features which passed

the 10% feature missingness threshold (for a combined total of 9506 features from C18 and HILIC chromatography) was imported to Mummichog. Features were ranked by their MWAS statistical significance. Pathways that were differentially enriched by location in features meeting an FDR-adjusted MWAS significance threshold of 0.05 were identified. All significantly different pathways were required to contain at least three mapped metabolites meeting the FDR threshold of 0.05. Similarly enriched pathways (by the same cutoff) were also identified. Analysis was performed with a mixed ion mode, with a mass tolerance of 5 p.p.m., with RT present, and with primary ions enforced.

Metabolomics–Exposomics integration analysis

The exposome–metabolome network analysis aimed to identify associations between environmental chemicals and metabolomic pathways that best characterize the bile content of patients with PSC by geographical location. This facilitates an understanding of the common and distinct composition of PSC bile at different geographical locations. Inputs to the analysis included the 92 environmental chemicals assayed and the 95 metabolomic pathways identified in pathway analysis, all of which were adjusted for age, sex, and duration of PSC. Pathways were represented by principal component 1 of all pathway metabolites.[6] The analysis was completed using xMWAS,[35] which provides an automated framework for integrative and differential network analysis. Pairwise integration between chemicals and metabolomic pathways was performed through a canonical sparse partial least squares (sPLS) regression analysis. All associations $|r| \geq 0.6$ and a Bonferroni-adjusted value of $P < 5.72 \times 10^{-6}$ (.05 divided by [92 chemicals × 95 pathways]) were retained and visualized using Cytoscape.[36] Communities of tightly correlated chemicals and pathways were detected by multilevel community detection.[37] The assumption underlying community detection is that communities comprised functionally related molecules.[35] Networks and communities were visualized to compare the associations of environmental chemicals and metabolomic pathways by the geographical site.

Results

Demographic and clinical characteristics

We summarize patient characteristics in Table 1. The sample comprised of 46% and 50% women in the US and Norway groups, respectively. Patients had a similar median age at PSC diagnosis (40 years of age in the USA and 37 years of age in Norway). There was no difference in the prevalence of IBD between cohorts. The most prescribed IBD medication in both cohorts was Mesalamine (Asacol). Furthermore, there were no differences in the rates of clinically important endpoints between the two cohorts (see comorbidities, Table 1). No patients had received a liver transplant before the time of bile collection. At the time of bile collection, the Norway patients were on average slightly younger and had lower rates of antidepressant medication, antihypertensive medication, and vitamin/supplement use. Overall, 69% of the patients in these samples had comorbid IBD, which is consistent with the literature.[32]

Exposomic analysis - EWAS

Nine environmental exposures identified using GC–HRMS were associated with geographical location (FDR < 0.20) (Figure 2; Supplementary Table SI). These include pesticide and insecticide compounds (alpha-BHC, bioallethrin, prothiofos), a PAH (fluorene), and five PCBs congeners. Levels of all of these chemicals were higher in patients in the USA compared with Norway. Hierarchical clustering showed no patient clustering patterns by sex, age group, duration of PSC, Crohn's disease status, ulcerative colitis status, ursodiol prescriptions, or vitamin supplementation. For example, men did not separate from women through clustering (and likewise, the remaining variables did not show separation by groups) (Figure 2C; see Supplementary Figure SI for clustering of all chemicals). The remaining 83 GC–HRMS-identified compounds had similar concentrations in the bile of patients with PSC in the USAand in Norway. The compound with the highest median concentration in both sites was di-n-butyl phthalate (DBP), followed by prothiofos. Pyriproxyfen had the third highest concentration in Norway patients, while Bioallethrin had the third highest concentration in the USA patients (Supplementary Table SI). The exploratory analysis assessing associations of environmental chemicals with IBD status controlling for location, sex, age, and duration of PSC demonstrated that no compounds were associated (FDR < 0.20) with IBD status, although six were associated at a nominal $P < .05$ (Supplementary Table SII). Ninety-seven of the 241 LC–HRMS-annotated environmental compounds met the quality control criteria of having <10% feature missingness. Of these 97, 22 were significantly different between the USA and Norway patients (Supplementary Table SIII). These annotated chemicals include drugs (Cotinine methonium ion), nutritive compounds (Vanillic acid), and environmental chemicals (Benzofuran).

Metabolomic differences by site

Following a 10% feature missingness threshold, 3526 features from the C18 chromatography column and 5978 features from the HILIC column were assessed for association with the geographical location through MWAS (described in the "Metabolome-wide association study" section). A total of 581 C18 features and 2562 HILIC features met an FDR threshold of 0.05, indicating that their intensity could be modeled through linear regression as a function of the geographical site, accounting for age, sex, and duration of PSC (Figure 3).

Metabolomic pathway analysis

Pathway enrichment analysis was performed using mummichog,[23] which infers pathway activities from a ranked list of mass spectrometry peaks that were derived through MWAS. Ranked features were imported into mummichog, then pathway enrichment of top-ranked features (FDR < 0.05 associating with geographical location) was calculated. Fifteen pathways were significantly enriched in top-ranked features by geographical location ($P < .05$), and 80 were similarly enriched between sites (Figure 4A for differential pathways; for all pathways, see Supplementary Table SIV). The differentially enriched pathways fall under broad categories of amino acid, glycan, carbohydrate, and vitamin/cofactor metabolism. Concentrations of putative metabolites localizing to these 15 differential pathways were both increased and decreased in the USA patients, depending on the metabolite (Figure 4B; Supplementary Table SV). Of these 15 pathways, compounds in the tyrosine metabolism pathway had the highest fold-change differences (both higher and lower) in patients across locations (Figure 4B).

Exposome–Metabolome integration analysis

The integrative network analysis was performed to characterize associations between identified environmental chemicals and metabolic pathways in bile and to compare these associations by geographical location (Figures 1 and 5). A canonical sPLS regression approach enabled pairwise integration of the 92 identified

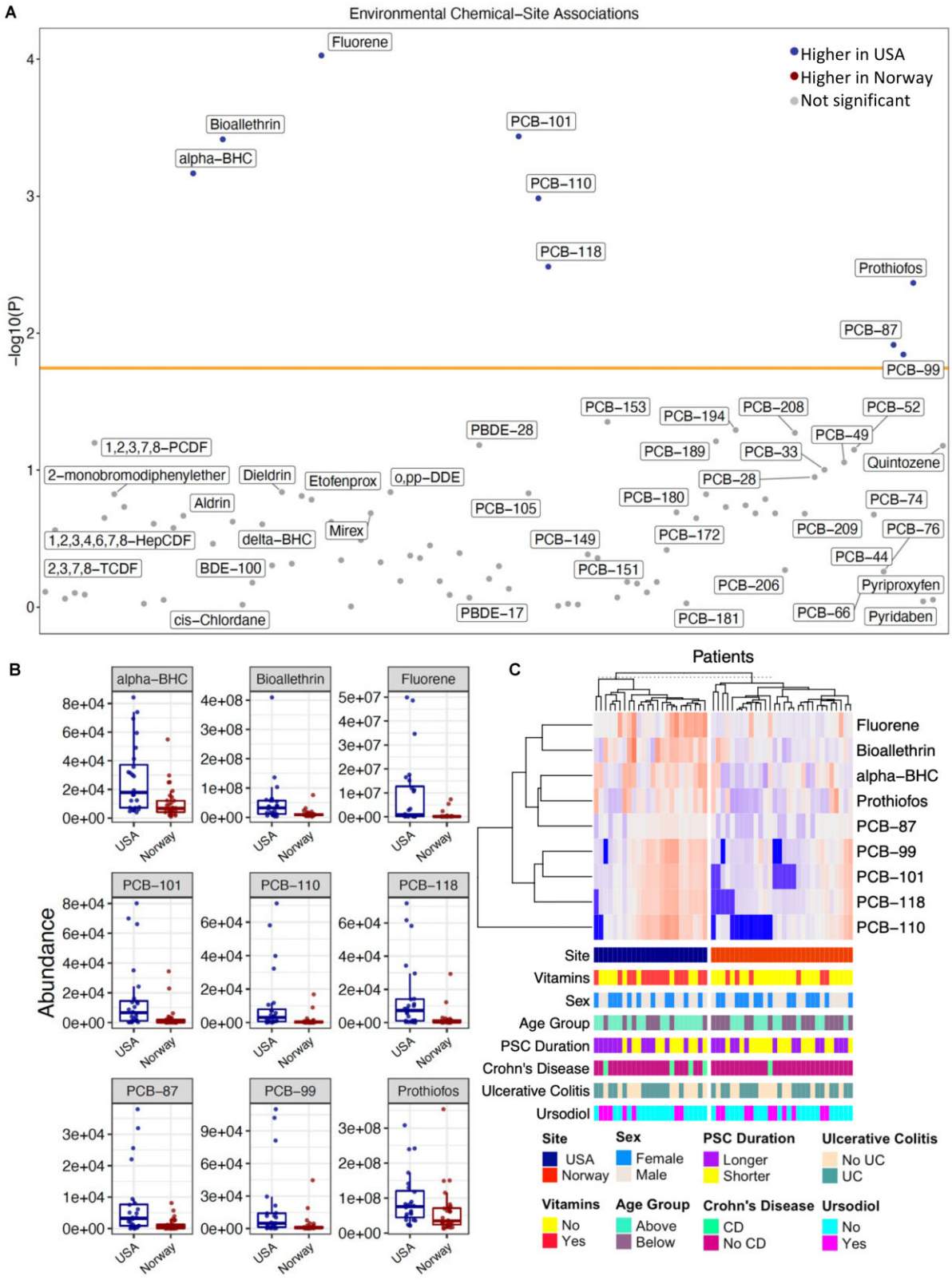

Figure 2. Environmental chemicals by geographical location. (A) Associations of the 92 environmental chemicals with the site. The compound number (1 through 92) is represented on the x-axis. Labels are provided as space allows (please see Supplementary Table S1 for a complete list of chemicals). (B) Abundance versus site for the nine exposures which associate with the site ($P < .05$) after adjusting for age, sex and duration of PSC. (C) Log2 transformed and normalized chemical intensities of the nine site-associated exposures (FDR <0.2) with hierarchical clustering by site (average linkage, Manhattan distance).

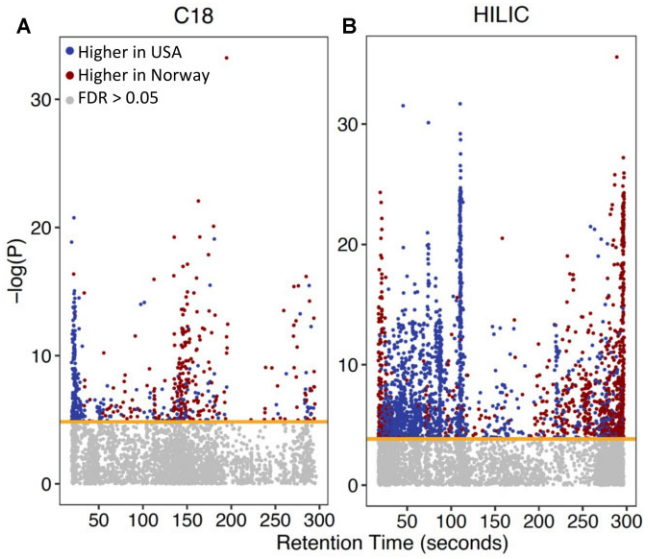

Figure 3. MWAS for associations of *m/z* features with geographical site for the (A) C18-negative HPLC column and (B) HILIC-positive HPLC column.

chemicals and 95 metabolic pathways and selection of those which best characterize bile of patients with PSC by site. Additionally, communities of highly associated pathways and chemicals within networks were detected.

Four communities comprising a total of 33 pathways were associated with one or multiple of six chemicals in the USA patients. The chemicals represented on the USA network include five, which are significantly higher in the USA than the Norway patients by EWAS (PCB-101, PBC-87, PBC-118, and bioallethrin), and one that was detected at comparable levels in the USA and Norway patients (2-monobromodiphenylether). The metabolic pathways associated with these chemicals included those with significantly different enrichment by geographical location (in the broad categories of amino acid metabolism, carbohydrate metabolism, and glycan biosynthesis and metabolism) and with similar concentrations.

Comparatively, fewer environmental chemicals are associated with fewer metabolic pathways in the Norway network. Only 3 communities comprising only 11 pathways were associated with one of three chemicals in Norway patients (Figure 5). PCB-118, PCB-101, and quintozene were retained in the Norway network. These were associated with glycan biosynthesis and metabolism and energy metabolism pathways, which were differentially enriched between sites, as well as pathways that were similar in the USA and Norway bile samples.

Four metabolic pathways were similarly enriched between the USA and Norway patients and associated with environmental chemicals in each sample. The specific chemical–metabolome associations differed by site. In the Norway sample, caffeine metabolism, n-glycan degradation, and glycosphingolipid biosynthesis (ganglioseries) were associated with PCB-101, while these pathways were associated with the bioallethrin and 2-monobromoeiphenlether community in the USA sample. Bile acid biosynthesis was associated with quintozene in the Norway sample and PCB-118 in the USA sample. Network statistics can be found in Supplementary Tables SVI and SVII.

Lastly, the nontargeted analysis study reporting tool was utilized to evaluate all study designs and reporting procedures (Supplementary Table SVIII).[38]

Discussion

This is the first comprehensive characterization of the bile exposome in patients with PSC. Characterization of bile in PSC is critical, as bile directly contacts the diseased bile ducts. Through state-of-the-art HRMS technology- and network-based analytical approaches, patients with PSC located in distinct geographical regions were found to have shared and differential environmental chemicals, endogenous metabolites, and chemical–metabolomic associations in bile. The derived chemical–metabolomic associations are an important step in understanding the biochemical changes that coincide with environmental chemical exposure in PSC, as they may reflect mechanisms toward disease pathogenesis or progression. Therefore, the present findings serve as a starting point that highlights key exposures and principles toward understanding the interplay between the environment and host in the bile of patients with PSC.

The MWAS found 3143 of 12 647 (~25%) features to differ between sites, and pathway analysis demonstrated that 15 of the 95 metabolomic pathways were differentially enriched by the geographical site. Thus, this first characterization of metabolomic content by geographical site suggests heterogeneity of bile metabolomic content in patients with PSC based purely on the geographical location. These differences may stem from different environmental exposures, lifestyle variance, or a combination of the two.

This work is the first to reveal the diverse range of environmental chemicals in human bile. Numerous human exposure assessment studies have demonstrated that these chemicals, especially the persistent contaminants, can be detected in various biospecimens and confer adverse effects in several tissues (eg, neurotoxicity and nephrotoxicity). We speculate that the chemicals detected in bile also will affect the liver (the primary site of biotransformation), the digestive system (the primary source of chemical ingestion through food and water), and the bile ducts (through direct contact). It is noteworthy to mention that many chemicals are subject to enterohepatic circulation mediated by the bile, which increases the RT and chemical burden in the liver, blood, digestive system, and bile ducts. Eighty-three environmental chemicals were detected at statistically similar concentrations in patients across the two geographic sites. At both sites, DBP had the highest median bile concentration. DBP is an endocrine disruptor that associates with splenic toxicity, obesity, and type II diabetes, with no current known associations with PSC.[39,40] Interestingly, DBP is used for enteric coating in certain formulations of mesalamine (Asacol, Asacol[HD]),[41] a drug used to treat IBD, the most common comorbidity in this population. DBP-containing IBD medications were the most prescribed IBD medications in both the USA and Norway samples. Given the known associations between DBP and disease and the high DBP bile concentrations in these samples, future investigations are warranted to study whether (1) DBP in bile contributes to the development of PSC and (2) IBD pharmacotherapy promotes high DBP bile concentrations.

The bile concentrations of five PCB congeners (PCB-87, PCB-99, PCB-101, PCB-110, PCB-118), three pesticide/insecticide compounds (bioallethrin, prothiofos, and alpha-BHC), and a PAH (fluorene) differed by location in these patients. For all of these, concentrations were higher in patients in the USA compared with Norway. The effect of higher environmental chemical concentrations appears to be increased crosstalk with metabolomic activity, represented through network analysis by the larger number of chemical–metabolomic associations in the USA patients

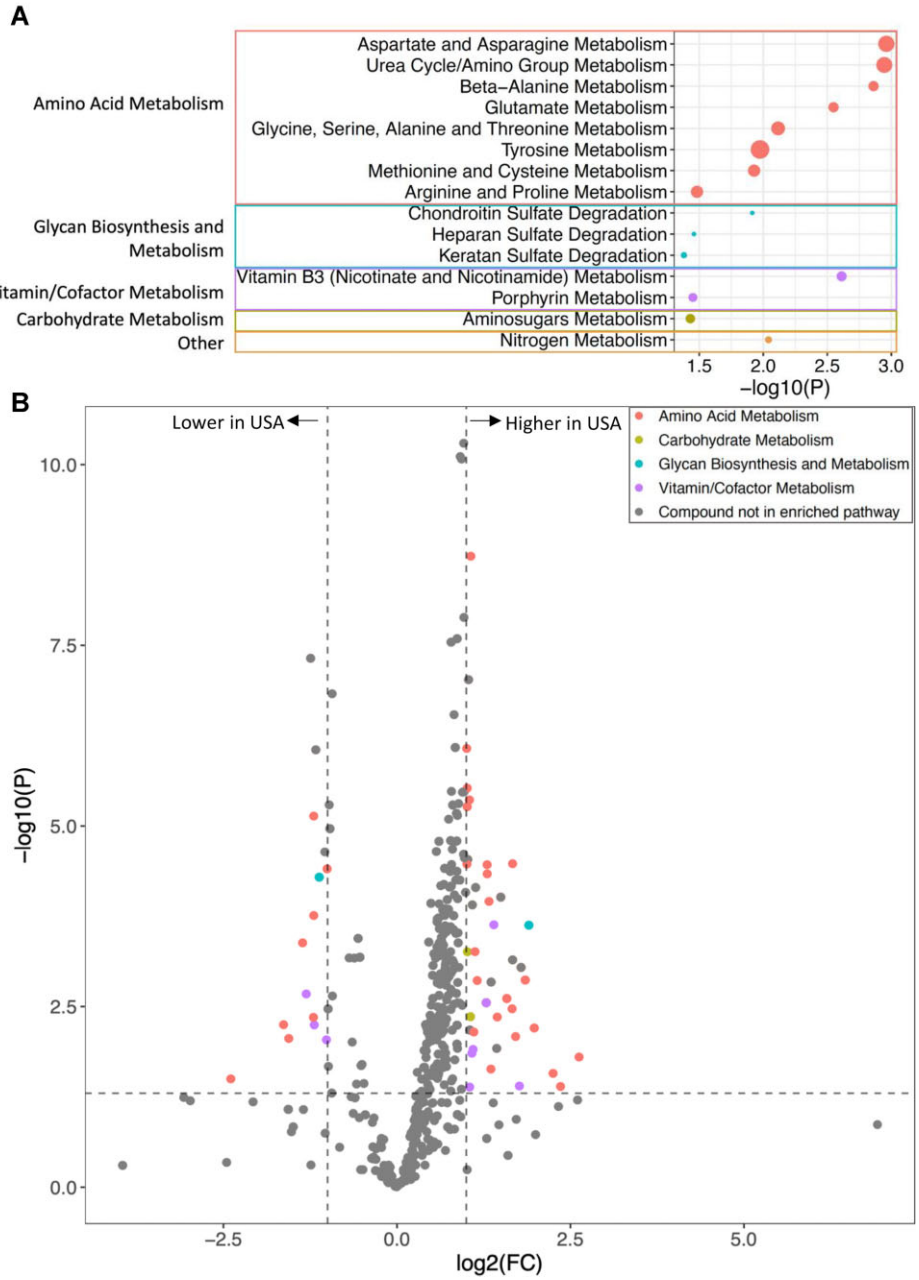

Figure 4. Pathway analysis for enrichment by geographical location. (A) Pathways significantly enriched by geographical location ($P < .05$). The size of the circles corresponds to the number of compounds mapping to that pathway. (B) Metabolites represented in differentially enriched pathways. Log2(FC): log2 fold-change, with higher values corresponding to higher metabolite concentrations in the USA compared with Norway.

compared with Norway. Thus, upon entry of environmental compounds into bile, the bile ducts encounter not only those environmental compounds but also all associated metabolites. Whether these exogenous agents, the associated endogenous metabolites, or the combination of the two directly harm the bile ducts should be explored in future functional experiments.

The network analyses enable assessment of the chemical–pathway associations which exist in patients at both geographical sites. No chemical–pathway associations observed in the USA patients were also observed in the Norway patients. However, pathways represented in the USA network (without their USA network-associated environmental chemicals) and environmental chemicals of the USA network (without their USA network-associated pathways) were observed in the Norway patients. Specifically, chemicals that were associated with metabolomic activity in both cohorts include PCB-118 and PCB-101. PCBs are highly stable organic chemicals that were widely manufactured in plasticizers, paints, and electrical equipment until they were banned by the Stockholm Convention on POPs. PCB-118 is known to promote the development of cholangiocarcinoma, hepatocholangioma, and hepatocellular adenoma in rats.[42,43] Cholangiocarcinoma is the most common malignancy in patients with PSC.[44,45] PCB-101 associates with fatty liver diseases.[46] Whether PBC-118 and PCB-101 promote the development of PSC, and whether this is mediated by metabolomic activity of pathways represented in the network analyses, warrant future investigation.

In the Norway cohort, PCB-118 most highly associated with two differentially enriched pathways, heparan sulfate degradation and

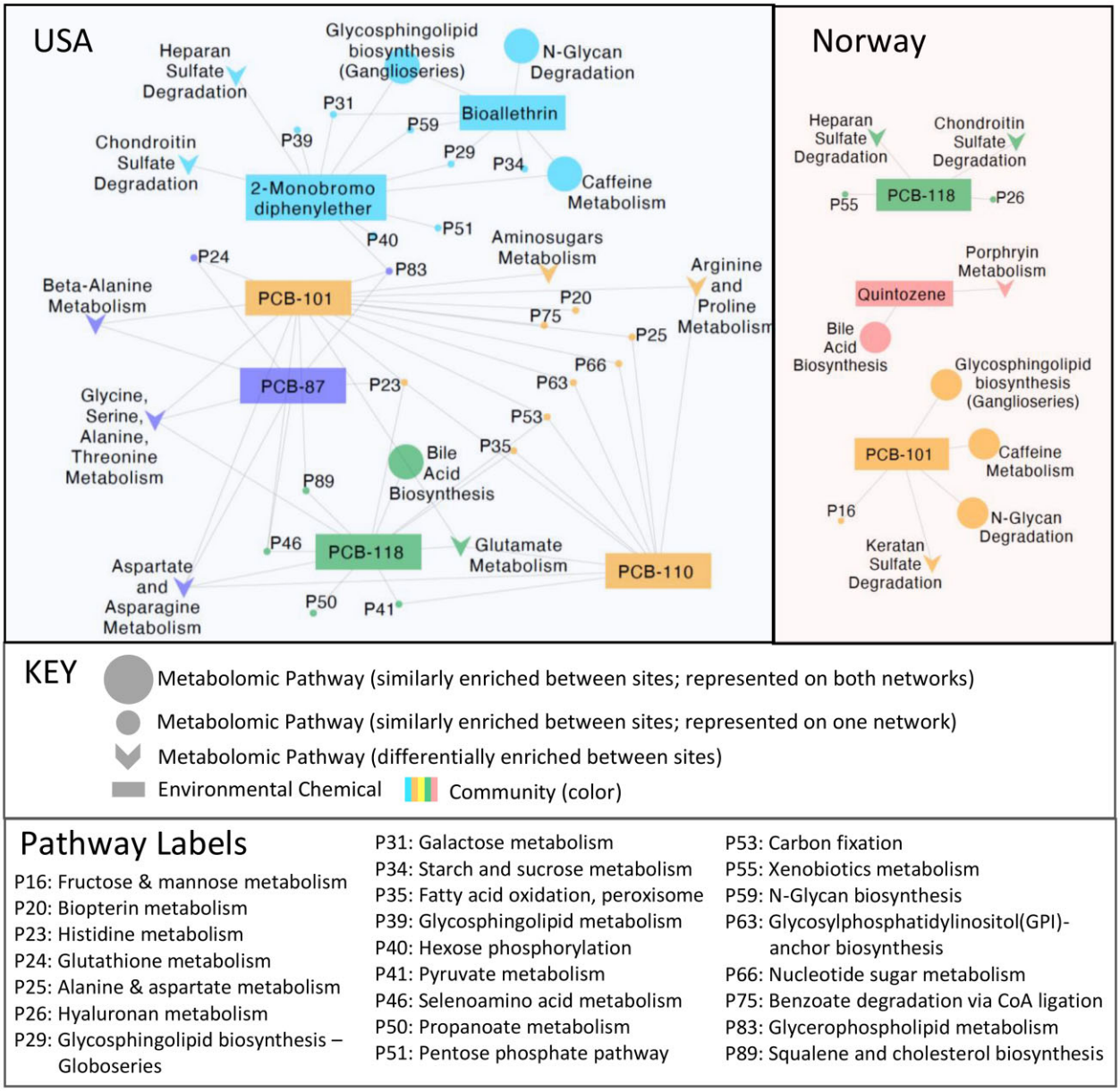

Figure 5. Multi-omics integration. Integrated networks of environmental chemicals and metabolomic pathways stratified by location. Arrow-shaped labeled pathways represent those that are differently enriched ($P < .05$) by the site. Large circular pathways represent those that are similarly enriched ($P > .05$) by the site and represented in both the USA and Norway networks. Pathways with smaller circles labeled as 'P#' are similarly enriched ($P > .05$) by the site and represented on either the USA or the Norway network. Pathway number corresponds to the pathway analysis results, ordered by the significance of differential enrichment between sites.

chondroitin sulfate degradation, both of which are glycan degradation pathways. PCB-101 most highly associated with one differentially enriched pathway, keratan sulfate degradation (an additional glycan degradation pathway), as well as N-glycan degradation. This contrasts with the USA cohort, where PCB-118 and PCB-101 associate broadly with a larger number of diverse metabolomic pathways. In the USA cohort, the glycan degradation pathways (heparan sulfate degradation, N-glycan degradation, chondroitin sulfate degradation) were associated most strongly with 2-monobromodiphenylether and bioallethrin. The differential chemical–pathway associations across networks may reflect differences in chemical concentrations or chemical–chemical interactions. Of note, however, is the fact that glycan degradation pathways were associated with one or multiple environmental chemicals in both samples of patients. This indicates that metabolomic activity in these pathways may have multifactorial chemical contributors dependent on chemical concentrations or chemical mixtures that these patients are exposed to. Glycans are complex oligosaccharides, which modify proteins, and glycan degradation is one of the major metabolic processes to shape the composition of the gastrointestinal microbiome.[47] The high comorbidity of PSC with IBD has led to accumulating evidence of altered gastrointestinal microbiome in the pathogenesis of PSC.[48-51] Given the relevance of glycans to PSC pathophysiology, the chemicals and chemical mixtures characterized in this work which may affect glycan degradation (PCB-101, bioallethrin, 2-Monobromodiphenylether) warrant additional investigation.

To assess whether the metabolic pathways represented in these networks were enriched in the plasma of an independent cohort of patients with PSC, comparisons were drawn between

the present analysis and a recent case–control plasma PSC study.[6] None of the compounds ($n = 12$), which significantly differentiated patients with PSC ($n = 80$) from healthy controls ($n = 40$) in the plasma study were assayed in the present work. This highlights the need to determine relevant biomarkers of interest to be explored in multiple physiological compartments (eg, bile, plasma, liver) in future studies.

There are limitations to this study. This study considered 92 environmental chemicals identified by GC–HRMS, providing the first such characterization of bile in patients with PSC. However, it is estimated that more than 100 000 chemicals are present in the environment[8] and that any given individual may have current or past exposures to thousands of chemicals. Therefore, there may be additional chemicals present in the bile of patients with PSC that are below current detection limits or were, due to a transient nature, not present at the time of sampling. Current technologies limit the extent of environmental chemical detection and must continue to evolve to enable large-scale assessments. Additionally, while PSC is a rare disease and collecting bile via ERCP is challenging, the sample size was relatively small. Larger cohorts are necessary to validate the characterized associations between environmental chemicals and metabolomic pathways. Given that this study included PSC cases only, it remains unclear if the presence of environmental and endogenous chemicals in bile fluid is causally or coincidentally related to liver disease. The inclusion of healthy controls requires the performance of an ERCP—an invasive procedure with no benefit and a real risk to the participant, conferring significant challenges to the collection of appropriate control samples. Additionally, these analyses are correlational in nature, and associations between chemicals and metabolic pathways do not necessarily imply causative effects. It is therefore possible that the differential chemical concentrations observed between geographical sites are mediated by sociodemographic factors not collected in the present work (eg, diet, physical activity, occupation, body fat percentage). Mechanistic studies in laboratory animals or *in vitro* systems are necessary to determine the cause–response relations between these molecules.

In conclusion, this novel study provides the first characterization of the exposome in the bile of patients with PSC. The study demonstrates that it is possible to measure dozens of environmental chemicals in human bile. The results show the heterogeneity of bile in PSC, with shared and variable endogenous and exogenous factors relating to geographical location. Higher concentrations of environmental chemicals in the USA cohort are associated broadly with endogenous metabolic pathways, suggesting functional crosstalk. Derived associations between glycan degradation pathways with environmental chemicals suggest a potential interaction of the gut microbiome with the metabolome and exposome in patients with PSC in a chemical concentration-dependent manner. Future case–control and longitudinal studies are warranted to further elucidate the endogenous and environmental contributors to PSC, which may ultimately guide necessary pharmacotherapy development in PSC.

Supplementary material

Supplementary material is available at *Exposome* online.

Data availability

The data underlying this article will be shared upon reasonable request to the authors (THK and KNL).

Authors' contributions

Caroline W. Grant (Conceptualization, Formal analysis, Methodology, Visualization, Writing—original draft), Brian D. Juran (Conceptualization, Data curation, Investigation, Project administration, Resources), Ahmad H. Ali (Data curation), Erik M. Schlicht (Resources), Jackie K. Bianchi (Resources), Xin Hu (Data curation, Methodology, Validation), Yongliang Liang (Data curation, Methodology, Validation), Zachery Jarrell (Data curation, Methodology, Validation), Ken Liu (Data curation, Methodology, Validation), YoungMi Go (Data curation, Methodology, Validation), Dean Jones (Conceptualization, Funding acquisition), Douglas I. Walker (Data curation, Methodology, Validation), Gary W. Miller (Conceptualization, Funding acquisition), Trine Folseraas (Data curation, Resources), Tom H. Karlsen (Conceptualization, Funding acquisition), Nicholas F. LaRusso (Conceptualization, Funding acquisition), Gregory J. Gores (Conceptualization, Funding acquisition), Arjun P. Athreya (Conceptualization, Funding acquisition, Writing—review and editing), and Konstantinos N. Lazaridis (Conceptualization, Funding acquisition, Writing—review and editing).

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Conflict of interest statement

None declared.

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