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Epigenetic disease markers in primary sclerosing cholangitis and primary biliary cholangitis—methylomics of cholestatic liver disease

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Abstract

Background: The epigenome, the set of modifications to DNA and associated molecules that control gene expression, cellular identity, and function, plays a major role in mediating cellular responses to outside factors. Thus, evaluation of the epigenetic state can provide insights into cellular adaptions occurring over the course of disease.

Methods: We performed epigenome-wide association studies of primary sclerosing cholangitis (PSC) and primary biliary cholangitis (PBC) using the Illumina MethylationEPIC Bead Chip.

Results: We found evidence of increased epigenetic age acceleration and differences in predicted immune cell composition in patients with PSC and PBC. Epigenetic profiles demonstrated differences in predicted protein levels including increased levels of tumor necrosis factor receptor superfamily member 1B in patients with cirrhotic compared to noncirrhotic PSC and PBC. Epigenome-wide association studies of PSC discovered strongly associated 5′-C-phosphate-G-3′ sites in genes including vacuole membrane protein 1 and SOCS3, and epigenome-wide association studies of PBC found strong 5′-C-phosphate-G-3′ associations in genes including NOD-like receptor family CARD domain containing 5, human leukocyte antigen-E, and PSMB8. Analyses identified disease-associated canonical pathways and upstream regulators involved with immune signaling and activation of macrophages and T-cells. A comparison of PSC and PBC data found relatively little overlap

Abbreviations: AMA, anti-mitochondrial antibodies; CCA, cholangiocarcinoma; CpG, 5′-C-phosphate-G-3′; EWAS, epigenome-wide association study; FDR, false discovery rate; GWAS, genome-wide association study; GWsig, genome-wide significance; HLA, human leukocyte antigen; IBD, inflammatory bowel disease; MHC, major histocompatibility complex; PBC, primary biliary cholangitis; PC, principal components; PSC, primary sclerosing cholangitis; SGsig, suggestive significance.

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at the 5′-C-phosphate-G-3′ and gene levels with slightly more overlap at the level of pathways and upstream regulators.

Conclusions: This study provides insights into methylation profiles of patients that support current concepts of disease mechanisms and provide novel data to inspire future research. Studies to corroborate our findings and expand into other -omics layers will be invaluable to further our understanding of these rare diseases with the goal to improve and individualize prognosis and treatment.

INTRODUCTION

Primary sclerosing cholangitis (PSC) and primary biliary cholangitis (PBC) are rare, immune-mediated liver diseases characterized by biliary damage and progressive fibrosis, which over time can lead to advanced cirrhosis and end-stage liver disease. In PSC, the biliary damage is primarily focussed on medium-sized and large-sized extrahepatic and intrahepatic ducts,[1] whereas in PBC, small intrahepatic ducts are affected.[2] The etiology and pathogenesis of both diseases are complex, resulting from some interacting combination of genetic and environmental factors, and remain relatively obscure.[3] Genome-wide association studies (GWAS) of PSC and PBC have identified numerous risk loci in both human leukocyte antigen (HLA) and non-HLA genes that highlight the strong role of the immune system in the genetic risk of these diseases. However, while many of the identified risk alleles do overlap with those found for other autoimmune diseases, there is little overlap between risk alleles for PSC and PBC, emphasizing the difference in immune mechanisms underlying disease development.[4]

While GWAS findings in PSC and PBC have been an important source for innovation to better understand important disease processes, additional approaches that are more amenable to evaluating disease progression and outcomes are needed. The epigenome, which is the constellation of modifications to DNA and associated molecules that control gene expression, cellular identity, and function, is an important mediator of the cellular response to environmental and stochastic factors.[5] As such, evaluation of epigenetic states can provide valuable insights into cellular adaptions occurring over the course of disease. Epigenome-wide association studies (EWAS) have been employed in the study of numerous complex diseases and have been particularly successful in the context of autoimmunity.[6] Many of these focus on the evaluation of cytosine methylation at 5′-C-phosphate-G-3′ (CpG) dinucleotide sequences, an important epigenetic mechanism that plays a key role in regulating gene expression and can be practically assessed at a genome scale.

Previous efforts to evaluate DNA methylation in PSC and PBC have been largely targeted and in small patient populations.[7] Recently, we reported that EWAS of liver tissue and blood of patients with PSC and PBC using the sequencing-based approach reduced representation bisulfite sequencing. Liver tissue from patients with PSC and PBC was characterized by extensive hypomethylation of genes involved with T cell activation and immune signalling relative to healthy donor controls.[8] In blood, differential methylation was largely split between hypomethylation and hypermethylation and enriched pathways included inositol phosphate metabolism (both diseases), IL-17 signaling (PBC), and TGF-β signaling (PSC).[9] While epigenome-wide approaches were used in these studies, sample sizes were small, and the use of reduced representation bisulfite sequencing limited downstream analytics. Here, we report an EWAS of a large cohort of patients with PSC and PBC and well-matched control groups using an array-based platform, the Illumina MethylationEPIC Bead Chip.

METHODS

Patients

Patients with PSC and PBC and controls without liver disease were selected from the PSC Scientific Community Resource.[10] Patients were diagnosed according to the American Association for the Study of Liver Diseases practice guidelines for PSC[11] or PBC,[12] and disease-specific characteristics were abstracted from patient medical records by experienced physicians. Cirrhosis was defined by (a) histopathological findings consistent with cirrhosis using the Ludwig histological staging system; (b) liver parenchymal changes on cross-sectional imaging compatible with cirrhosis along with physical and laboratory findings compatible with cirrhosis; (c) presence of portal hypertension not caused by non-liver–related etiologies; and/or (d) severe liver dysfunction and portosystemic shunting as documented by presence of HE. Written informed consent was obtained from all participants and the study conformed

to the ethical guidelines of the Declarations of Helsinki and Istanbul as reflected by a priori approval of the Mayo Clinic Institutional Review Board.

Methylome profiling

Methylome profiling was performed using the Infinium HD Methylation assay and MethylationEPIC Bead Chip (Illumina) according to the manufacturer's instructions. Briefly, Qubit quantitated DNA was bisulfite treated using the EZ-96 DNA Methylation kit (Zymo Research). Modified DNA samples were denatured with NaOH, neutralized, and underwent isothermal whole-genome amplification in the presence of RPM and MSM reagents (Illumina). Amplified DNA was enzymatically fragmented, precipitated with isopropanol, and resuspended in RA1 buffer (Illumina). Prepared samples were robotically loaded onto the BeadChips using a Freedom EVO unit (Tecan) and hybridized at 48°C overnight. Following hybridization, BeadChips were washed with RA1 reagent (Illumina) to remove unhybridized and nonspecifically hybridized DNA, and then underwent single-base extension and staining. The BeadChips were then imaged using an iScan instrument (Illumina) and analyzed using GenomeStudio software (Illumina).

Methylation data pre-processing and quality control

Raw Infinium MethylationEPIC .dat files were processed using R package "ChAMP" (v2.16.2).[13] Methylated and unmethylated signal intensities were extracted, and average beta, which represents the methylation ratio at a CpG site, was calculated. Samples with over 10% of CpG sites having detection *p*-value greater than 0.01 and CpG sites with detection *p*-value greater than 0.01 in more than 10% of samples were excluded. CpG sites with a bead count less than 3 in more than 10% of samples or sites whose probe sequence maps to multiple genomic locations[14] were also excluded. Data points still missing were imputed using the KNN algorithm. The 2 probe types on MethylationEPIC arrays have different biases in methylation level measurement.[15] To correct these biases, we applied BMIQ normalization[16] before further analyses.

Sample level quality assessment was performed using technical replicates of positive and negative control samples with very high or low methylation embedded across plates and principal components analysis for CpGs in autosome, gender, or all chromosomes together regarding sample annotations: processing batch, plate, and gender. Replicates across plates were highly correlated (median correlation coefficient 0.985), no clusters were associated with the sample plate, and samples were clearly segregated into clusters of males and females.

Phenotypic estimations using DNA methylation data

DNA methylation-derived "epigenetic clocks" contrasting chronological with biological age have received interest as age acceleration is associated with a variety of clinical phenotypes and outcomes.[17,18] In this study, we focussed on the variable *AgeAccelGrim*.[19] Methylation profiles can be used to approximate proportions of the main immune cell types in blood samples, providing insight and a means to adjust linear regression models in EWAS. We estimated the proportions of T-cells (CD4 and CD8), B-cells, natural killer cells, monocytes, and granulocytes using the Houseman method.[20] *AgeAccelGrim* calculation and immune cell estimation were performed using the "New Methylation Age Calculator" (http://dnamage.genetics.ucla.edu/new), and comparisons of these values between groups were performed using the Kruskal-Wallis test. Methylation profiles can also be used to predict levels of some circulating proteins. To this end, we used the tool MethylDetectR to generate Epi Scores for 109 proteins.[21,22] and compared levels in patients with PSC and PBC and subgroups using the Kruskal-Wallis test.

Epigenome-wide association study (EWAS)

EWAS analyses were performed for PSC, PBC, and disease subgroups wherein CPG associations were tested in R using the cpg.assoc function from the CpGassoc package. For each CPG, this function fits a linear model with the CPG beta value as the response variable and group status (eg, patients with PSC vs. PSC controls) as the covariate of interest, with adjustment for age, gender, and epigenetically determined cell fractions. The cpg.assoc function provided unadjusted *p*-values, genomic control adjusted *p*-values, and false discovery rate (FDR) values. We conservatively elected to use the genomic control adjusted *p*-values to evaluate the significance of association, with CpGs having $p < 9.0 \times 10^{-8}$ considered genome-wide significant (GWsig)[23] and $p < 1.0 \times 10^{-5}$ considered to have suggestive significance (SGsig). Smoking and BMI were also considered in the model but did not considerably impact CpG significance and thus were not included. Significant CpGs were checked for inclusion in other EWAS studies using the EWAS catalog (http://ewascatalog.org).[24] Principal components (PC) were generated using the most variable 25% of CpGs on chromosomes 1–22 for all subjects and tested for associations between patient subgroups using the Kruskal-Wallis test.

Pathway and upstream regulator analysis

CpGs achieving SGsig or GWsig were mapped to genes using annotations provided by Illumina, and the resulting gene lists were used to perform canonical pathway and upstream regulator analysis using ingenuity pathway analysis (QIAGEN Inc., https://qiagenbioinformatics.com/products/ingenuity-pathway-analysis).[25] Pathways with FDR $p < 0.05$ and upstream regulators with $p < 0.001$ were considered significant. Venn diagrams were prepared using the tool available at https://bioinformatics.psb.ugent.be/webtools/Venn/.

RESULTS

Patient characteristics

A total of 474 patients with PSC and 484 PSC controls were available for analysis following QC (Table 1 and Supplemental Table S1, http://links.lww.com/HC9/A979). The groups were well-matched in age, gender, and race. Patient characteristics such as female gender, age of diagnosis, and concurrent inflammatory bowel disease (IBD) were consistent with previous reports.[1] Approximately 20% of the patients had undergone colectomy before sample collection, and an additional 17 patients received one in follow-up. Further, 75 of the 474 patients with PSC were cirrhotic at the time of sample collection, 32 of whom were decompensated, and an additional 60 patients developed decompensated cirrhosis in follow-up. A small percentage of the patients had been diagnosed with cholangiocarcinoma (CCA) before sample collection, and 23 of the patients developed CCA in follow-up.

A total of 291 patients with PBC and 291 PBC controls were available for analysis following QC (Supplemental Table S1, http://links.lww.com/HC9/A979). Demographic and clinical characteristics of these participants are provided in Table 1. The groups were well-matched in age, gender, and race. Among the patients, female gender, age of PBC diagnosis, and positivity for anti-mitochondrial antibodies (AMA) were consistent with that of the at-large PBC population.[26] Approximately 15% of the patients with PBC were cirrhotic at the time of sample collection, half of which were decompensated. An additional 18 patients developed decompensated cirrhosis in follow-up.

Epigenetic age acceleration

Biological age acceleration has been demonstrated in numerous diseases, including PSC.[27] We used

TABLE 1 Characteristics of patients and controls

	PSC	Control	*p*	PBC	Control	*p*
n	474	484	—	291	291	—
Age (y), Median (IQR)	49.8 (34.5–61.2)	50.2 (34.5–61.5)	0.935	61.4 (53.9–68.0)	61.1 (54.0–68.3)	0.970
Female gender, n (%)	182 (38.4)	184 (38.0)	0.947	259 (89.0)	259 (89.0)	> 0.999
Caucasian race, n (%)	448 (94.9)	463 (95.7)	0.456	281 (96.6)	284 (97.6)	0.624
Hispanic ethnicity, n (%)	0 (0)	7 (1.5)	0.016	4 (1.4)	2 (0.7)	0.686
Age diagnosis (y), Median (IQR)	40.3 (27.6-52.3)	—	—	53.7 (46.0-60.8)	—	—
Disease duration[a] (y), Median (IQR)	5.6 (2.3-11.0)	—	—	6.1 (2.4-11.0)	—	—
Cirrhotic at sample	—	—	—	—	—	—
Nondecompensated, n (%)	43 (9.1)	—	—	22 (7.6)	—	—
Decompensated, n (%)	32 (6.8)	—	—	23 (7.9)	—	—
Cirrhosis in follow-up	—	—	—	—	—	—
Nondecompensated, n (%)	20 (4.2)	—	—	7 (2.4)	—	—
Decompensated, n (%)	60 (12.7)	—	—	18 (6.2)	—	—
OLT in follow-up, n (%)	48 (10.1)	—	—	11 (3.8)	—	—
AMA positive PBC, n (%)	—	—	—	248 (85.2)	—	—
Small duct PSC, n (%)	32 (6.8)	—	—	—	—	—
PSC with IBD, n (%)	378 (79.7)	—	—	—	—	—
Colectomy at sample, n (%)	104 (21.9)	—	—	—	—	—
Colectomy in follow-up, n (%)	17 (3.6)	—	—	—	—	—
CCA at sample, n (%)	15 (3.2)	—	—	—	—	—
CCA in follow-up, n (%)	23 (4.9)	—	—	—	—	—

[a]Disease duration of PSC or PBC at the time of sample collection. Categorical variables are compared using Fisher exact test, and continuous variables are compared using the Kruskal-Wallis test.

P-values < 0.05 are considered significant.

Abbreviations: AMA, anti-mitochondrial antibodies; CCA, cholangiocarcinoma; IBD, inflammatory bowel disease; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis.

AgeAccelGrim, the age-adjusted variation of *DNAm GrimAge*,[19] to evaluate epigenetic age acceleration in PSC and PBC. This tool has proven superior to previous epigenetic clocks[19] but has not yet been used in the context of cholestatic liver disease. We found *AgeAccelGrim* to be consistently elevated in patients with PSC compared to controls across the age spectrum (Figure 1A and B) and in patients with cirrhotic compared to noncirrhotic PSC (Figure 1C). Findings in PBC were similar, with a consistent elevation of *AgeAccelGrim* noted across the range of participant ages (Figures 1D and E). However, the elevation of *AgeAccelGrim* in patients with cirrhosis compared to those without cirrhosis did not reach statistical significance in PBC (Figure 1F).

We also compared *AgeAccelGrim* values between patients belonging to important subphenotype groups in PSC and PBC (Supplemental Figure S1, http://links.lww.com/HC9/A980). Notably, there was little difference when comparing patients with PSC with and without IBD ($p = 0.8214$) and patients with PSC with and without colectomy ($p = 0.9152$). Patients with PSC with CCA at the time of sample collection had higher values than those without, although the small number of patients with CCA limited statistical power ($p = 0.1747$). In addition, patients with PSC with small duct disease had lower *AgeAccelGrim* values than those without, but again, this difference fell short of significance ($p = 0.0673$). Finally, there was no difference between patients with AMA+ and AMA-PBC ($p = 0.8398$). Values for all *AgeAccelGrim* comparisons are provided in Supplemental Table S2, http://links.lww.com/HC9/A979.

Epigenetic estimation of immune cell types

We used the method described by Houseman et al[20] to estimate the proportions of immune cells in our samples. Patients with PSC had reduced levels of CD4 T-cells, CD8 T-cells, and natural killer cells and increased levels of B-cells and monocytes relative to controls (Figure 2A). These trends were more pronounced in patients with cirrhotic PSC compared to

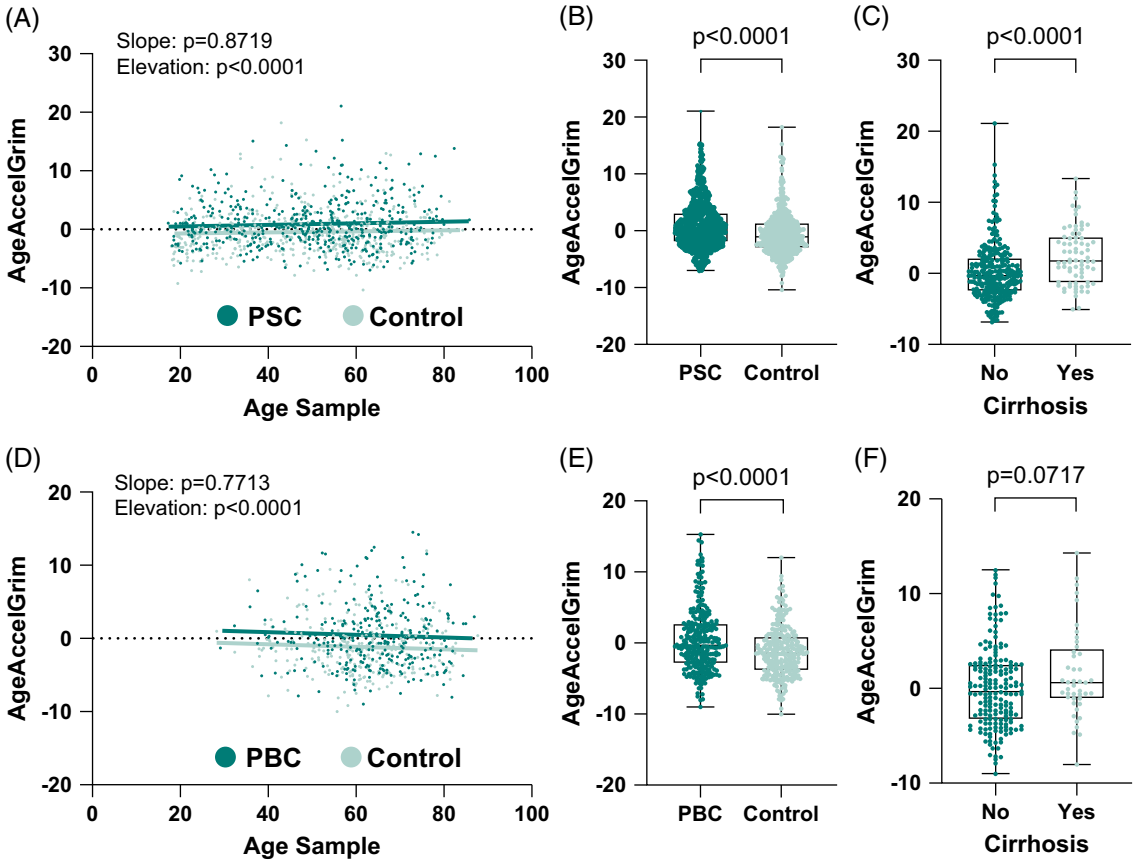

FIGURE 1 AgeAccelGrim scores in patients with PSC and PBC. (A) AgeAccelGrim by age at sample collection in patients with PSC and controls. (B) AgeAccelGrim score comparison between patients with PSC and controls. (C) AgeAccelGrim score comparison between patients with cirrhotic and noncirrhotic PSC. (D) AgeAccelGrim by age at sample collection in patients with PBC and controls. (E) AgeAccelGrim score comparison between patients with PBC and controls. (F) AgeAccelGrim score comparison between patients with cirrhotic and noncirrhotic PBC. Individual data points are shown. Box and whiskers indicate the median, IQR, and minimum/maximum. Slope and elevation are compared using linear regression. AgeAccelGrim comparison *p*-values are calculated using the Kruskal-Wallis rank sum test. Abbreviations: PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis.

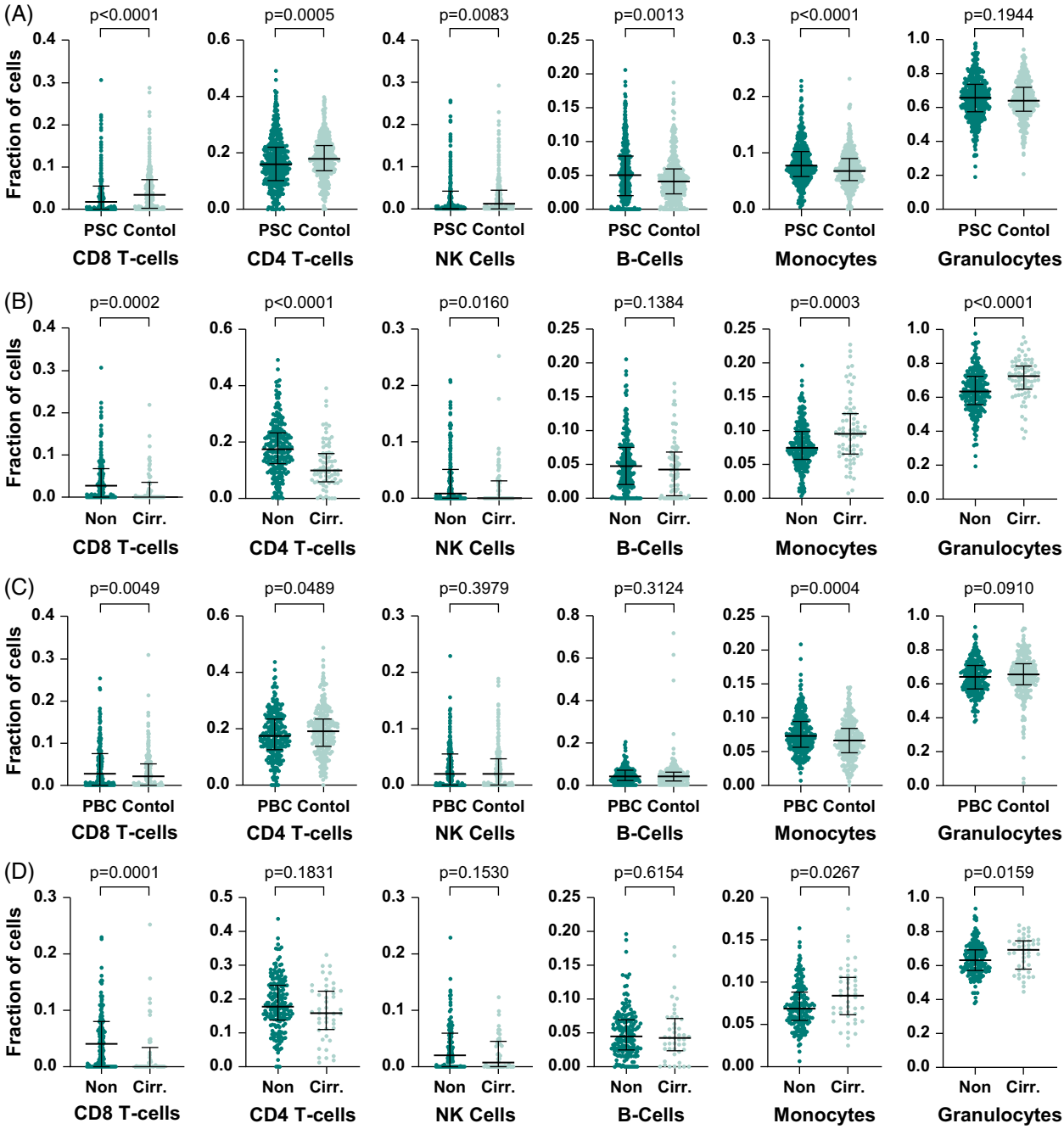

FIGURE 2 Epigenetic immune cell composition in patients with PSC and PBC. (A) Comparison of patients with PSC and controls. (B) Comparison of patients with cirrhotic and noncirrhotic PSC. (C) Comparison of patients with PBC and controls. (D) Comparison of patients with cirrhotic and noncirrhotic PBC. Individual data points are shown. Whiskers indicate median and interquartile range. *p*-values are calculated using the Kruskal-Wallis rank sum test. Abbreviations: PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis.

those who were not cirrhotic at the time of sample collection (Figure 2B). PBC similarly demonstrated an increased proportion of monocytes and reduced CD4 T-cells compared to controls but differed by having an increased fraction of CD8 T-cells (Figure 2C). Notably, 4 PBC controls were found to be outliers having high B-cell and/or low granulocyte proportions (Figure 2C) and this was considered in further analyses. Patients with cirrhotic PBC had a significantly lower proportion of CD8 T-cells and higher levels of monocytes and granulocytes than those without cirrhosis (Figure 2D). Values for comparisons presented in Figure 2 are provided in Supplemental Table S3, http://links.lww.com/HC9/A979.

We also compared estimated immune cell proportions between patients belonging to phenotypic subgroups of PSC and PBC (Supplemental Figure S2, http://links.lww.com/HC9/A981, and Supplemental Table S3, http://links.lww.com/HC9/A979). Patients with PSC with colectomy before sample collection had a significantly reduced

proportion of CD4 T-cells and increased monocytes and granulocytes compared to patients with no colectomy. Patients with PSC with small duct disease had increased levels of CD4 T-cells compared to patients with nonsmall duct. Notably, despite the small sample size, patients with PSC with CCA at the time of sample collection had significantly reduced proportions of T-cells (both CD4 and CD8) and B-cells and increased proportions of monocytes and granulocytes compared to patients who did not have CCA. We found no difference in estimated cell proportions between patients with PSC with or without IBD or between patients with PBC based on AMA status.

Epigenetic estimation of circulating protein levels

We used MethylDetectR to generate Epi Scores for 109 proteins[21,22] and compared levels in patients with PSC and PBC and subgroups. We found 52 of the proteins to be associated with PSC (FDR $p < 0.05$), 29 of which were increased and 23 decreased in patients with PSC relative to controls (Supplemental Table S4, http://links.lww.com/HC9/A979). Epi Score values for the top 6 PSC-associated proteins are shown in Figure 3A, illustrating decreased values for lactotransferrin and increased values for colony stimulating factor 3, C9, CD163, C-reactive protein, and Fc epsilon receptor 2. Comparison of Epi Scores between patients with PSC with and without cirrhosis found 67 of the proteins to be associated with cirrhosis (FDR $p < 0.05$), with 27 decreased and 40 increased in patients with cirrhosis compared to those without cirrhosis (Supplemental Table S4, http://links.lww.com/HC9/A979). The top 6 cirrhosis-associated proteins are shown in Figure 3B, illustrating further increases in levels of PSC-associated proteins C9 and C-reactive protein in patients with cirrhosis. In PBC, 40 of the proteins were associated with disease (FDR $p < 0.05$), 33 of which were increased, and 7 of which were decreased in patients compared to controls (Supplemental Table S5, http://links.lww.com/HC9/A979). Epi Scores for the top 6 PBC-associated proteins show increased levels of c-x-c motif chemokine ligand 11, CD163, fc gamma receptor 3A, CD48, c-x-c motif chemokine ligand 9, and cytotoxic and regulatory T cell molecule in patients relative to controls (Figure 3C). A total of 15 proteins were associated with cirrhosis in patients with PBC (FDR $p < 0.05$), 9 of which were increased and 6 decreased in patients with cirrhosis compared to those without cirrhosis (Supplemental Table S5, http://links.lww.com/HC9/A979). None of the top 6 cirrhosis-associated proteins in PBC directly overlapped with the top 6 PBC proteins, although tumor necrosis factor receptor superfamily member 1B, B2M, and c-x-c motif chemokine ligand 10 CpGs were highly associated with PBC in the case-control analysis (Figure 3D).

Evaluation of estimated protein levels in PSC phenotypic subgroups identified 5 proteins associated with IBD status, 10 proteins associated with colectomy, 15 proteins associated with small duct disease, and 24 proteins associated with CCA (FDR $p < 0.05$) (Supplemental Table S4, http://links.lww.com/HC9/A979). Of note, despite the low sample numbers, the strongest protein associations among the subgroups were with CCA and included increased levels of C-reactive protein, retinoic acid receptor responder 2, S100 calcium binding protein A9, and C9 and decreased levels of Notch receptor 1 and CD6 in patients with PSC with CCA compared to those without (Supplemental Figure S3, http://links.lww.com/HC9/A982). No proteins were found to be associated with AMA positivity in patients with PBC (Supplemental Table S5, http://links.lww.com/HC9/A979).

Epigenome-wide association study

The PSC EWAS identified 80 CpG sites achieving GWsig ($p < 9.0 \times 10^{-8}$) and an additional 437 CpGs demonstrating SGsig ($p < 1.0 \times 10^{-5}$). Highly associated findings included multiple CpGs near the genes vacuole membrane protein 1 and SOCS3 on chromosome 17, TNFSF10 on chromosome 3, and TNFSF4 on chromosome 1 (Table 2, Figure 4A and B). The majority of PSC-associated CpGs, including 87.5% of GWsig CpGs and 79.2% of SGsig CpGs, were found to be hypomethylated in patients compared to controls (Figure 4C). Notably, the top 15 PSC-associated CpGs have previously been reported as significant in EWAS of Crohn's disease and IBD.[28,29] A complete list of PSC-associated CpGs, along with basic annotation, is provided in Supplemental Table S6, http://links.lww.com/HC9/A979. The PBC EWAS found 74 CpGs reaching GWsig and an additional 331 CpG sites with SGsig. Highly associated loci included multiple CpGs near the genes NOD-like receptor family CARD domain containing 5 (NLRC5) on chromosome 16, B2M on chromosome 15, and HLA-E and PSMB8 in the major histocompatibility complex (MHC) region of chromosome 6 (Table 2, Figures 4D and E). Removal of 4 PBC controls with outlier levels of estimated B-cell and/or granulocyte cell proportions did not change the findings, so they were kept in further analyses. As with PSC, the majority of PBC-associated CpGs were hypomethylated in patients compared to controls, including 94.6% of GWsig CpGs and 83.1% of SGsig CpGs (Figure 4F). In contrast to PSC, many PBC-associated CpGs were previously reported as significant in EWAS of Sjogren's syndrome,[30] rheumatoid arthritis,[31] and antibody production in systemic lupus erythematosus.[32] A complete list of PBC-associated CpGs is provided in Supplemental Table S7, http://links.lww.com/HC9/A979.

To explore how disease severity and sub-phenotypes influence the methylome, we performed a series of

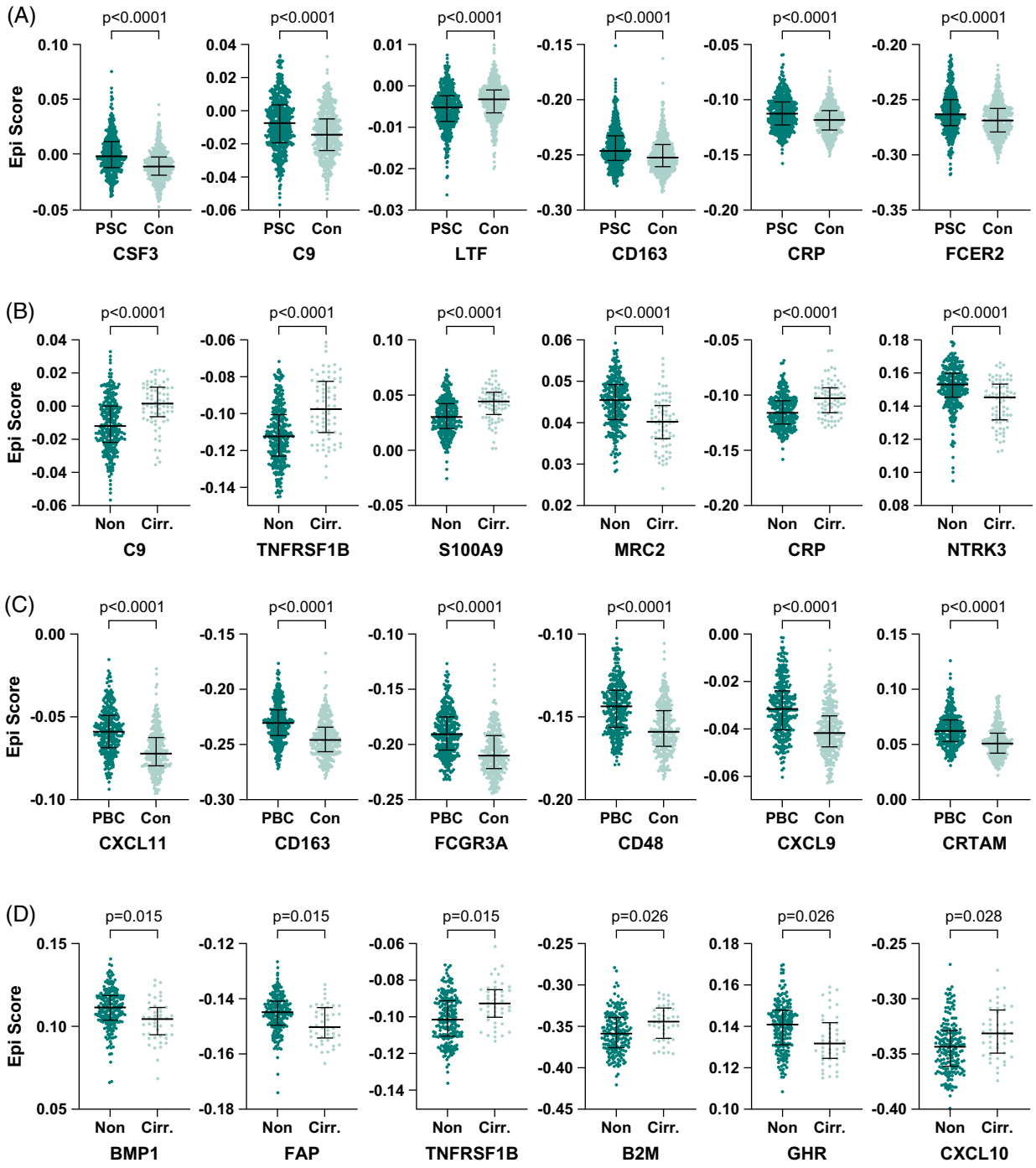

FIGURE 3 Epigenetically predicted protein levels in patients with PSC and PBC. (A) Comparison of patients with PSC and controls. (B) Comparison of patients with cirrhotic and noncirrhotic PSC. (C) Comparison of patients with PBC and controls. (D) Comparison of patients with cirrhotic and noncirrhotic PBC. The top 6 associations are shown for each comparison. Individual data points are shown. Whiskers indicate median and IQR. *p*-values are calculated using the Kruskal-Wallis rank sum test with corrections for false discovery using the Benjamini-Hochberg Procedure. Abbreviations: PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis.

analyses comparing PCs and sub-group–based EWAS. In PSC, the first 3 PCs were significantly different between patients with cirrhosis and those without cirrhosis, while in PBC, only the first PC demonstrated a significant difference (Supplemental Figure S4, http://links.lww.com/HC9/A983). Sub-group EWAS comparing patients with cirrhotic and noncirrhotic PSC and PBC identified no CpGs that reached GWsig; however, 26 CpGs in PSC and 10 CpGs in PBC were associated with cirrhosis at SGsig. Notably, the majority of these CpGs were found to be hypomethylated in patients with cirrhosis compared to those without cirrhosis (23/26 in PSC and 8/10 in PBC) (Supplemental Table S8, http://links.lww.com/HC9/A979). Comparison of PCs between

TABLE 2 Top CpGs identified in EWAS of PSC and PBC

Disease	CpG	chr	Position[a]	% Diff[b]	Direction[c]	gc.p-val	Gene	MeWAS traits[d]
PSC	cg16936953	17	57915665	6.50	Hypo	2.3×10^{-17}	VMP1	CD[e], IBD[f]
	cg12054453	17	57915717	6.46	Hypo	2.9×10^{-17}	VMP1	CD[e], IBD[f]
	cg19748455	17	76274856	4.31	Hypo	5.0×10^{-16}	—	CD[e]
	cg00840791	19	16453259	9.33	Hypo	5.7×10^{-16}	—	CD[e]
	cg11047325	17	76354934	7.57	Hypo	7.4×10^{-16}	SOCS3	CD[e]
	cg18942579	17	57915773	4.88	Hypo	1.8×10^{-15}	VMP1	CD[e,f]
	cg18181703	17	76354621	4.29	Hypo	3.1×10^{-15}	SOCS3	CD[e], IBD[f], T2D[g]
	cg01409343	17	57915740	3.87	Hypo	9.1×10^{-15}	VMP1	CD[e], IBD[f]
	cg13343932	17	76355061	5.88	Hypo	2.0×10^{-14}	SOCS3	CD[e]
	cg03067296	17	76274577	5.29	Hypo	7.7×10^{-14}	—	CD[e]
	cg00303773	17	17752575	3.49	Hypo	2.3×10^{-12}	TOM1L2	CD[e]
	cg12992827	3	101901234	5.05	Hypo	3.8×10^{-12}	—	CD[e], IBD[f]
	cg27023597	17	57918262	3.27	Hypo	5.7×10^{-12}	MIR21	CD[e]
	cg00382138	4	110723299	4.36	Hypo	7.2×10^{-12}	CFI	CD[e]
	cg01059398	3	172235808	2.87	Hypo	7.9×10^{-12}	TNFSF10	CD[e]
PBC	cg07839457	16	57023022	10.67	Hypo	1.2×10^{-21}	NLRC5	CD[e], IBD[f], RA[h], SLE[i]
	cg16411857	16	57023191	4.36	Hypo	2.9×10^{-17}	NLRC5	CD[e], IBD[f], RA[h], Sjo[j], SLE[i]
	cg27537252	15	45006400	5.55	Hypo	7.9×10^{-16}	B2M	—
	cg06511149	19	30469222	2.38	Hypo	6.6×10^{-15}	URI1	—
	cg05475649	15	45007015	3.48	Hypo	3.5×10^{-14}	B2M	—
	cg08818207	6	32820355	3.07	Hypo	4.7×10^{-14}	TAP1	CD[e], IBD[f], RA[h], Sjo[j]
	cg11594821	6	30458601	2.55	Hypo	3.1×10^{-13}	HLA-E	Sjo[j]
	cg21979287	15	45007854	2.38	Hypo	4.1×10^{-13}	B2M	–
	cg08099136	6	32811251	3.18	Hypo	1.3×10^{-12}	PSMB8	Sjo[j], SLE[i]
	cg09313918	1	54232631	2.36	Hypo	1.5×10^{-12}	NDC1	—
	cg09858955	2	58135951	5.54	Hypo	1.7×10^{-12}	VRK2	—
	cg01309328	6	32811253	2.74	Hypo	2.6×10^{-12}	PSMB8	Sjo[j], SLE[i]
	cg23235965	6	30459540	2.07	Hypo	4.6×10^{-12}	HLA-E	—
	cg12828896	15	45005365	5.85	Hypo	4.5×10^{-10}	B2M	—
	cg22107533	15	45028083	2.44	Hypo	8.3×10^{-10}	TRIM69	Sjo[j]

[a]GRCh37.
[b]% Difference in mean methylation between controls and cases.
[c]Direction of methylation difference in patients compared to controls.
[d]Traits with significant findings in other MeWAS.
[e]PMID:30779925.
[f]PMID:27886173.
[g]PMID:26095709.
[h]PMID:23334450.
[i]PMID:26192630.
[j]PMID:26857698.
Abbreviations: CD, Crohn's disease; CpG, 5′-C-phosphate-G-3′; EWAS, epigenome-wide association study; IBD, inflammatory bowel disease; MeWAS, methylome-wide association study; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis; RA, rheumatoid arthritis; Sjo, Sjogren's disease; SLE, antibody production in systemic lupus erythematosus; T2D, type 2 diabetes; VMP1, vacuole membrane protein 1.

patients with PSC based on IBD status and type did not uncover significant differences, and sub-group EWAS identified only 3 suggestive-level CpG associations, indicating that concurrent IBD might not have a strong influence on the epigenetic landscape in PSC. Comparison of patients with PSC with and without colectomy before sample collection identified a significant difference in PC3, although like IBD, sub-group EWAS identified only 2 marginally suggestive associations (Supplemental Figure S5, http://links.lww.com/HC9/A984, and Supplemental Table S8, http://links.lww.com/HC9/A979). In PBC, PCs did not differ between patients who were AMA positive and AMA negative, while in PSC, a significant difference in PC1 between patients with small duct disease compared to typical disease primarily affecting larger bile ducts was identified. Notably, patients with PSC diagnosed with CCA before sample collection had significant differences in PC1 and PC3 compared to

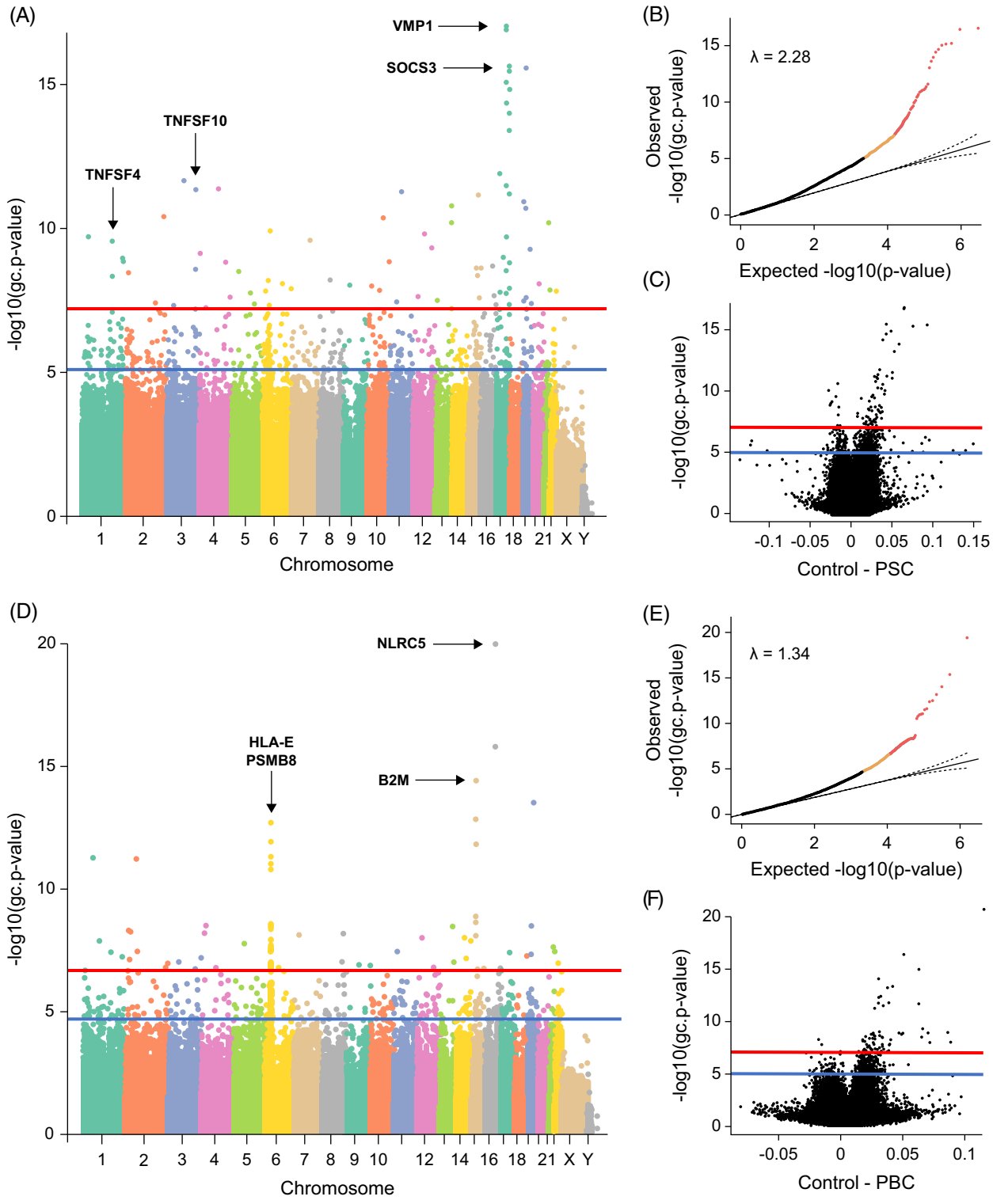

FIGURE 4 Epigenome-wide association study (EWAS) of PSC and PBC. (A) Manhattan plot showing results of PSC EWAS. (B) Q-Q plot of PSC EWAS results. (C) Volcano plot of PSC EWAS results. (D) Manhattan plot showing results of PBC EWAS. (E) Q-Q plot of PBC EWAS results. (F) Volcano plot of PBC EWAS results. The suggestive significance ($p < 1.0 \times 10^{-5}$) cutoff is indicated by the blue line, and the genome-wide significance ($p < 9.0 \times 10^{-8}$) cutoff is indicated by the red line. Abbreviations: EWAS, epigenome-wide association study; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis.

patients without CCA (Supplemental Figure S6, http://links.lww.com/HC9/A985). Unfortunately, EWAS for AMA, small duct disease, and CCA was precluded because of limited numbers of patients in these subgroups.

Ingenuity pathway analysis pathway and upstream regulator analysis

The 517 PSC-associated CpGs and 405 PBC-associated CpGs mapped to 323 and 268 genes, respectively (Supplemental Table S9, http://links.lww.com/HC9/A979). These gene lists were used to perform canonical pathway and upstream regulator analyses using ingenuity pathway analysis software. In PSC, 39 pathways were found to be associated with disease (FDR $p < 0.05$), including pathways involved with macrophage activation, immune signaling, and cancer mechanisms (Figure 5A and Supplemental Table S10, http://links.lww.com/HC9/A979). As well, 35 upstream regulators were associated with PSC ($p < 0.001$), including cytokines IFNG and IL2 and transcription regulators ZNF512B and SP1 (Figure 5B and Supplemental Table S11, http://links.lww.com/HC9/A979). In PBC, 38 pathways were associated with disease (FDR $p < 0.05$), including pathways involved with antigen presentation and immune signaling (Figure 5C and Supplemental Table S12, http://links.lww.com/HC9/A979). Moreover, 58 upstream regulators were associated with PBC ($p < 0.001$), including cytokines IFNG and IL27 and transcriptional regulators NLRC5 and STAT1 (Figure 5D and Supplemental Table S13, http://links.lww.com/HC9/A979).

A review of the genes underlying pathway enrichment results suggested that numerous associated HLA genes in both PSC and PBC could significantly impact the findings. Thus, we repeated the pathway and upstream regulator analyses, removing the HLA genes from the gene lists. While pathways and upstream regulators did change ranks, and some moved from significant to nonsignificant, the composition of the lists stayed largely the same, highlighting the importance of immune signalling in both diseases (Supplemental Figure S7, http://links.lww.com/HC9/A986, and Supplemental Tables 14–17, http://links.lww.com/HC9/A979).

Comparing and contrasting the blood methylomes of PSC and PBC

To better understand the similarities and differences in the blood methylomes of PSC and PBC, we performed some simple comparisons. At the CpG level, we found the majority of associated CpGs to be unique to either PSC or PBC, with 38 total CpG sites overlapping, 3 of which achieved GWsig in both diseases (Figure 6A). This finding largely carried over to the gene level, with 49 genes overlapping between PSC and PBC, only 1 of which, ABCG1, was GWsig in both diseases (Figure 6B). Notably, ABCG1 and the underlying CpG site, cg06500161, are reported to be associated with lipid-associated traits in numerous studies (ewascatalog.org).[24] The PSC and PBC gene lists contain 6 (PSC) and 7 (PBC) HLA genes, 1 of which, HLA-B, overlaps between diseases. Notably, the HLA genes in PSC are primarily HLA class II, while in PBC, they are primarily HLA class I (Figure 6C). Approximately one-third of the associated pathways overlapped between PSC and PBC, including pathways involving Ag presentation and T-cell activation. Pathways specific to PSC included STAT3 and IL-9 signaling and those specific to PBC included pathways involved with interferon signaling and T-cell exhaustion (Figure 6D and Supplemental Table S18, http://links.lww.com/HC9/A979). Finally, a comparison of the top 20 upstream regulators in PSC and PBC found 25% to overlap, including interferon alpha and gamma, whereas regulators including IL2 were specific to PSC and STAT1 was specific to PBC (Figure 6E).

DISCUSSION

In this first, large EWAS of PSC and PBC, we report novel data on epigenetic-derived estimates of traits such as biological age, immune cell composition, protein levels, and strongly associated CpG sites with each disease in genes across the genome. These valuable epigenetic data provide novel insights and perspectives to better understand and evaluate disease using DNA from peripheral blood, particularly in liver diseases, where access to the affected tissue is extremely limited.

Efforts to establish and understand the correlation between methylation profiles and age are predicated on predictive ability for age-related outcomes such as time-to-death and overall life span. We used one of the newer epigenetic age predictors, *AgeAccelGrim*,[19] and found evidence of age acceleration in patients with PSC and PBC relative to control groups across the age spectrum and increased values in patients with cirrhosis compared to patients without cirrhosis. This finding is consistent with a previous report focussed on patients with PSC that used an earlier estimator, DNAm age,[17] and found values to be increased in patients with PSC and that higher levels of age acceleration were associated with PSC-related events in patients.[27] No such previous study in PBC has been reported. Aging often includes declines in innate and adaptive immune responses, driven in part by chronic inflammation and immune senescence, and thus, these mechanisms have been proposed to contribute to age acceleration observed in many diseases. However, to what extent these inflammatory factors contribute to the age

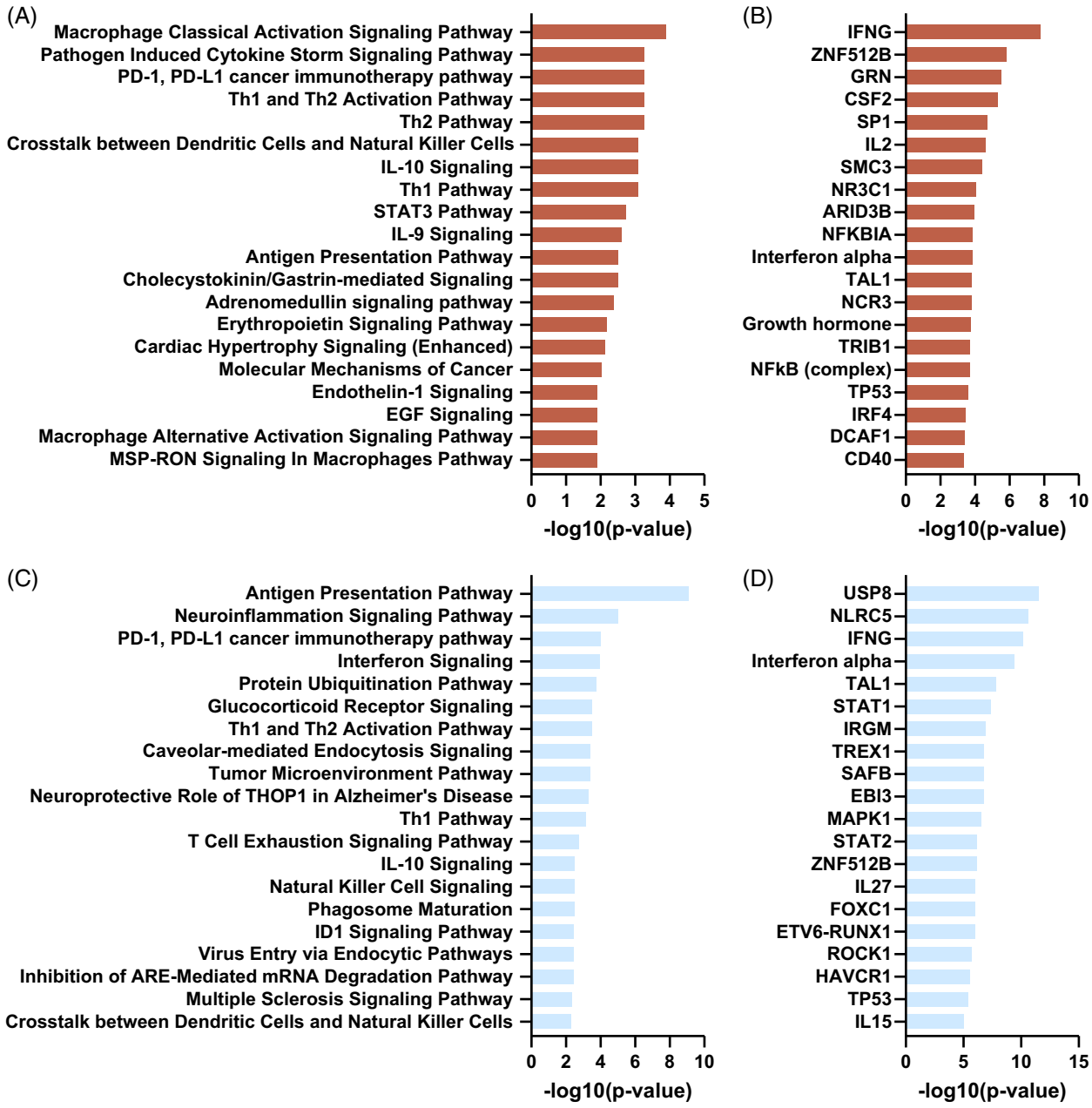

FIGURE 5 IPA pathway and upstream regulator analyses in PSC and PBC. (A) Top 20 PSC-associated canonical pathways. (B) Top 20 PSC-associated upstream regulators. (C) Top 20 PBC-associated canonical pathways. (D) Top 20 PBC-associated upstream regulators. Benjamini-Hochberg corrected *p*-values shown for pathway analyses and nominal *p*-values are shown for upstream regulators. Abbreviations: IPA, ingenuity pathway analysis; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis.

acceleration estimated in previous and current tools using methylation profiles remains unclear.[33]

Epigenetic prediction of cell type composition is limited in scope, particularly when compared to current and emerging single-cell technologies. However, it does provide a valuable and accepted basis to correct for cell composition variability in EWAS.[34] The primary finding in our study was that CD4+ T-cell proportions were decreased, and monocytes increased in patients with PSC and PBC compared to control groups. Whereas CD8+ T-cell proportions were decreased in PSC but increased in PBC. These findings are somewhat supported by a single-cell immunoprofiling study of PBC, which found higher levels of monocytes in patients compared to controls but did not find a difference in levels of overall CD8+ T-cells.[35] To our knowledge, no studies reporting on broad peripheral immune cell composition have been reported for PSC.

Prediction of circulating protein levels using methylation data is a recent advancement that could prove useful in the evaluation of immune-mediated diseases, considering that changes to the methylome in response

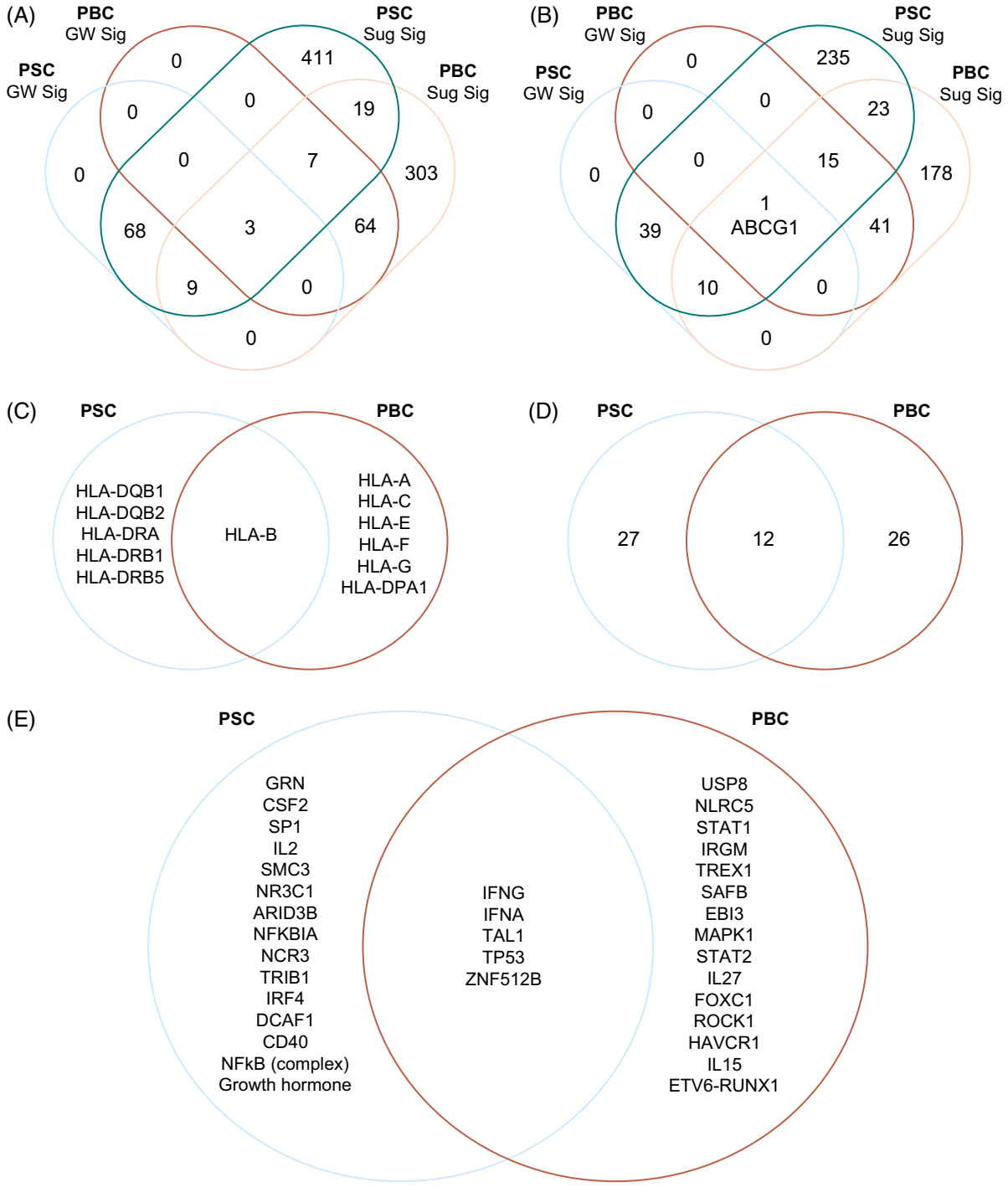

FIGURE 6 Comparison of PSC and PBC results using Venn diagrams. (A) CpGs demonstrating GW ($p < 9.0 \times 10^{-8}$) and sug ($p < 1.0 \times 10^{-5}$) significance in PSC and PBC EWAS. (B) Genes identified by GW and sug significance CpGs in PSC and PBC EWAS. (C) HLA genes identified by genome-wide or suggestive significance CpGs in PSC and PBC EWAS. (D) IPA canonical pathways (Benjamini-Hochberg corrected $p < 0.05$) in PSC and PBC. (E) IPA upstream regulators (top 20) in PSC and PBC. Abbreviations: CpG, 5′-C-phosphate-G-3′; EWAS, epigenome-wide association study; GW, genome-wide; HLA, human leukocyte antigen; IPA, ingenuity pathway analysis; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis; sug, suggestive.

to inflammation are likely to be more stable than direct protein measurements, which can vary significantly over time.[22] We used the tools available through MethylDetectR[21,22] to generate Epi Scores reflecting levels of 109 proteins and found 67 to significantly differ in PSC and 40 to differ in PBC compared to controls. While the percentage of proteins associated with disease seems high, it is not surprising considering that the protein set is enriched for potential involvement in inflammation and immunity. One compelling finding

was tumor necrosis factor receptor superfamily member 1B, which was among the top 3 increased proteins in patients with cirrhosis compared to patients with noncirrhotic PSC and PBC and highly significant in the PSC and PBC case-control analyses. tumor necrosis factor receptor superfamily member 1B encodes TNFR2, one of the 2 TNFα receptors. The soluble form of TNFR2 acts as a decoy receptor by binding soluble and membrane-bound TNFα, preventing downstream signalling. Notably, an increased level of soluble TNFR2 has predictive value in some tumors, inflammatory diseases, and kidney disease.[36,37] Another interesting finding was that B2M was also increased in patients with PSC and PBC relative to controls and in patients with cirrhosis compared to those without cirrhosis. Increased levels of circulating B2M have long been appreciated in numerous diseases, such as cancer and many chronic liver diseases, including PBC.[38] Whether or not epigenetically derived estimates of traits such as biological age or protein levels will lead to clinical benefits for patients with PSC and PBC remains unclear, but our study suggests they are a promising avenue for future research.

Our EWAS of PSC found 80 CpG sites achieving GWsig, the majority of which were hypomethylated compared to controls. Notably, 34 of the 80 sites, including 19 of the top 20, were associated with Crohn's disease and/or IBD in previous EWAS,[28,29] suggesting some level of shared pathogenesis and consistent with the strong overlap between PSC and IBD. Among the most highly associated genes were vacuole membrane protein 1, an ER-localized protein that plays important roles in autophagosome formation and ER communication with other organelles; SOCS3, a cytokine suppressor that blocks STAT activation through binding to JAK; TNFSF4 (aka OX-40L), a T-cell costimulatory molecule expressed on Ag-presenting and endothelial cells; and TNFSF10 (aka TRAIL), a death receptor ligand that induces apoptosis of cells expressing its cognate receptors that has been previously studied in the context of PSC.[39] A further 437 CpGs mapping to 273 additional genes achieved SGsig and included 5 MHC class II genes (HLA-DQB1, -DQB2, -DRA, -DRB1, and -DRB5) as well as the MHC class I gene HLA-B. This is notable considering the strong genetic association of PSC with HLA alleles.[4] Associated pathways included those involved with macrophage and T-cell activation, the STAT3 pathway, and cytokine/chemokine signalling, which is in line with current knowledge of mechanisms thought to be involved with PSC.[1] Top upstream regulators contained many inflammatory mediators, including IL2, which enforces previous assertions of the likely role of IL2 in PSC.[1]

The PBC EWAS identified 74 CpGs reaching GWsig, and as with PSC, the majority were hypomethylated relative to controls. Notably, 13 of the 79 CpGs, including 6 of the top 20, were associated with Sjogren's syndrome in a previous EWAS,[30] which is notable considering the strong relationship between Sjogren's and PBC.[40] Many highly associated genes are involved with Ag presentation and the MHC I pathway, including NLRC5, a key transcriptional regulator of MHC I; PSMB8, a subunit of the immunoproteasome responsible for protein degradation to MHC I compatible peptides in cells experiencing proinflammatory signalling; TAP1, a protein involved with peptide transport from the cytoplasm to the ER for loading to MHC I molecules; and B2M, a component of the MHC I molecule. Additionally, 4 HLA class I genes (HLA-A, -B, -C, and -E) and the HLA class 2 gene HLA-DPA1 were among the 57 genes identified using GWsig CpGs. Another 331 CpG sites mapping to an additional 211 genes reached SGsig and included PSMB9, the other immunoproteasome-specific subunit, as well as 2 more MHC class I genes, HLA-F and HLA-G. Associated pathways involving Ag presentation, interferon signalling, and T-cell activation were among the most significant and are consistent with the current understanding of PBC pathogenesis.[2] Top upstream regulators included NLRC5, which is not surprising given the associated class I genes, and IFNG, which supports previous assertions that IFNG plays an important role in PBC pathogenesis.[41]

Comparison of EWAS results between PSC and PBC found relatively little overlap at the CpG and gene levels, with 4.8% of SGsig and 2.0% of GWsig CpGs, and 11.0% of SGsig and 0.9% of GWsig genes common to the 2 diseases. The only gene that achieved GWsig in both PSC and PBC was ABCG1, a cholesterol efflux pump thought to control cellular sterol homeostasis that is active in a wide range of tissues and in the immune system and is reported in numerous blood-based EWAS of various lipid traits (ewascatalog.org).[24] While speculative, this finding may point to a link between the immune system and disrupted sterol metabolism due to underlying cholestasis in PSC and PBC. The GWsig genes did somewhat overlap with SGsig genes, with 20.0% of PSC and 26.3% of PBC GWsig genes overlapping with SGsig genes in the other disease. Among these were 2 microRNA (miR) gene clusters with immunoregulatory functions, the miR-17-92 gene cluster on chromosome 13 consisting of 7 miR genes[42] and the miR-15a/16-2 gene cluster on chromosome 3 consisting of 2 miR genes.[43] These demonstrate the potential involvement of miRs in PSC and PBC immunobiology. An additional 23 genes with SGsig overlapped between PSC and PBC and included PVT1 and ETS1, which have been reported as candidate genes in PBC GWAS, and CLEC16A, which has been reported in GWAS of both PSC and PBC.[4] Moreover, 1 SGsig gene in PSC was previously identified in PSC GWAS (FOXP1), and 3 such genes in PBC were previously reported in PBC GWAS

(SH2B3, ST8SIA4, and IRF5).[4] While our study was not designed to unravel mechanisms underlying these observations, they do point towards overlap between genetic and epigenetic processes in PSC and PBC.

There are several limitations to our study. First, the retrospective and cross-sectional nature implies association but does not establish causation. Also, the direction of effect on gene expression cannot be determined using only methylation data, and we cannot establish whether the differential methylation was present before the disease and had an impact on disease risk or is the result of underlying disease processes such as chronic inflammation, limiting mechanistic interpretation. However, differential methylation seen in PSC and PBC does represent significant disease hallmarks that might prove useful by informing the direction of pre-clinical research and in advancing approaches to disease prognostication. For example, strongly associated EWAS genes could become the focus of animal studies, or predicted variables based on methylation profiles (eg, epigenetic age and predicted proteins) could be incorporated into the development of next-generation prognostic scores.

In conclusion, we report the first, large, comprehensive EWAS study of patients with PSC and PBC that provides valuable new insights into large-scale methylation profile differences, which support current concepts of disease mechanisms and provide novel data to inspire future hypothesis-driven research. Similar-sized studies seeking to corroborate our findings and expand into other -omics layers are underway and will be very valuable to further our understanding of these rare diseases with the goal to improve and individualize prognosis and treatment.

AUTHOR CONTRIBUTIONS

Brian D. Juran had major roles in conceptualization, formal analysis, project administration, writing the original draft, and revising the manuscript. Bryan M. McCauley, Elizabeth J. Atkinson, and Zhifu Sun performed formal analysis. Erik M. Schlicht and Jackie K. Bianchi obtained patient resources. Jason M. Vollenweider and Hong Ye performed experimental investigation. Nicholas F. LaRusso and Gregory J. Gores reviewed and edited the final manuscript. Konstantinos N. Lazaridis conceived the study, acquired funding, supervised the project, and reviewed and edited the final manuscript.

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CONFLICTS OF INTEREST

The authors have no conflicts to report.

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