Promoter hypermethylation of the AE2/SLC4A2 gene in PBC

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Graphical abstract

Highlights
• Patients with PBC have higher AE2 CpG methylation in upstream AE2a and/or AE2b2/AE2b1 promoter regions in liver and PBMCs.
• Combined methylation rates of 2 minimal CpG-clusters in the liver and 1 minimal CpG-cluster in PBMCs specifically distinguished PBC from normal and diseased controls.
• Methylation rates of AE2 promoter regions inversely correlated with levels of respective AE2 mRNAs in liver and PBMCs.
• Alternate AE2b2/AE2b1 promoter regions were found to be densely methylated in both normal and diseased PBMC samples.

Lay summary
Primary biliary cholangitis (PBC) is a chronic immune-associated cholestatic liver disease with unclear complex/multifactorial etiopathogenesis affecting mostly middle-aged women. Patients with PBC exhibit reduced expression of the AE2/SLC4A2 gene. Herein, we found that AE2 promoter regions are hypermethylated in the liver and peripheral blood mononuclear cells of patients with PBC. This increased methylation is associated with downregulated AE2-gene expression, which might contribute to the pathogenesis of PBC. Therefore, novel epigenetic targets may improve treatment in patients with PBC who respond poorly to current pharmacological therapies.

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Promoter hypermethylation of the AE2/SLC4A2 gene in PBC

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Background & Aims: Patients with primary biliary cholangitis (PBC) exhibit reduced AE2/SLC4A2 gene expression in the liver and peripheral blood mononuclear cells (PBMCs). AE2 encodes a Cl⁻/HCO₃⁻ exchanger involved in biliary bicarbonate secretion and intracellular pH regulation. Reduced AE2 expression in PBC may be pathogenic, as AE2-knockout mice reproduce characteristic PBC features. Herein, we aimed to identify CpG-methylation abnormalities in AE2 promoter regions that might contribute to the reduced gene transcription in PBC livers and PBMCs.

Methods: CpG-cytosine methylation rates were interrogated at 1-base pair resolution in upstream and alternate AE2 promoter regions through pyrosequencing of bisulphite-modified genomic DNA from liver specimens and PBMCs. AE2a and alternative AE2b1 and AE2b2 mRNA levels were measured by real-time PCR. Human lymphoblastoid-T2 cells were treated with 5-aza-2’-deoxycytidine for demethylation assays.

Results: AE2 promoters were found to be hypermethylated in PBC livers compared to normal and diseased liver specimens. Receiver operating characteristic (ROC) curve analysis showed that minimal CpG-hypermethylation clusters of 3 AE2a-CpG sites and 4 alternate-AE2b2-CpG sites specifically differentiated PBC from normal and diseased controls, with mean methylation rates inversely correlating with respective transcript levels. Additionally, in PBMCs a minimal cluster of 3 hypermethylated AE2a-CpG sites distinguished PBC from controls, and mean methylation rates correlated negatively with AE2a mRNA levels in these immune cells. Alternate AE2b2/AE2b1 promoters in PBMCs were constitutively hypermethylated, in line with absent alternative mRNA expression in diseased and healthy PBMCs. Demethylation assays treating lymphoblastoid-T2 cells with 5-aza-2’-deoxycytidine triggered AE2b2/AE2b1 expression and upregulated AE2a-promoter expression.

Conclusions: Disease-specific hypermethylation of AE2 promoter regions and subsequent downregulation of AE2-gene expression in the liver and PBMCs of patients with PBC might be critically involved in the pathogenesis of this complex disease. © 2019 The Author(s). Published by Elsevier B.V. on behalf of European Association for the Study of the Liver (EASL). This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Introduction
Primary biliary cholangitis (PBC), formerly named primary biliary cirrhosis,1 is a chronic cholestatic liver disease mostly affecting middle-aged women, in which portal mononuclear infiltrates result in progressive damage and destruction of interlobular bile ducts.2–4 Thus far, the etiopathogenesis of PBC remains elusive. The disease is regarded as a complex multifactorial disease that may be triggered by environmental factors in individuals with genetic predisposition.5 Genome-wide-association studies (GWAS)7–10 and dense fine-mapping association studies11 have related susceptibility to PBC with genetic variations in genes pertinent to immunity like HLA type II, IL12A, IL12RB2, IL21 and IL21R genes among others. Certainly, the disease is strongly associated with autoimmune phenomena, such as the presence of autoreactive T cells in portal infiltrates and high-titer serum antimitochondrial autoantibodies (AMA) that recognize antigens at the inner mitochondrial membrane. Notwithstanding these features of autoimmunity in PBC, the therapeutic regimes with potent immunosuppressants have shown little efficacy, which is particularly intriguing if compared with the substantial benefit obtained in most patients with early PBC undergoing therapy with ursodeoxycholic acid (UDCA).12–16 Since the hydrophilic bile acid UDCA is known to induce bicarbonate-rich cholestasis, we have postulated that primary or secondary abnormalities in the mechanisms responsible for biliary bicarbonate secretion might have a pathogenic role in PBC.17–21 Indeed, positron-emission-tomography studies showed that untreated patients with PBC failed to increase biliary bicarbonate in response to secretin, while treatment with UDCA for a few months could reverse this defect.13

In humans, secretin-stimulated biliary bicarbonate secretion is crucial for adequate bile modifications along the biliary tract.21–24 Bicarbonate secretion occurs at the apical membrane of bile-duct cells via electroneutral Na⁺-independent Cl⁻/HCO₃⁻ anion exchange (AE), which can be stimulated by cAMP, the second messenger for secretin signal transduction.25–27 Previously, we reported that bile-duct cells isolated from PBC patients exhibit defective cAMP-stimulated AE activity,28 and that PBC livers have diminished AE2 expression at the luminal membrane of the biliary epithelium.19 Also, we reported that AE2 mRNA levels are decreased in liver biopsies and peripheral blood mononuclear

Keywords: AE2 chloride/bicarbonate exchanger; cholestasis; DNA methylation; epigenetic repression; primary biliary cholangitis/cirrhosis.

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cells (PBMCs) from patients with PBC. The notion that diminished AE2 might be involved in PBC pathogenesis received definite support from our findings in AE2-knockout mice, which exhibit characteristic features resembling PBC.

Conventional genotyping of selected AE2/SLC4A2 tag single-nucleotide polymorphisms (rs2069443, rs2303933, rs2303937, and rs2303941) in Japanese patients with PBC revealed associations with disease susceptibility and/or anti-centromere antibody production. But in Caucasian patients, no association between variations in AE2/SLC4A2 and PBC susceptibility has been reported, though single-nucleotide polymorphism analyses across this gene have found 2 variants influencing AMA status.

Intron 2 of the AE2b2 promoter regions (located within upstream promoter sequences (see Table S2). AE2a, AE2b1, and for GAPDH as internal control. For calculation, we used the relative quantification (ΔΔCt) method, which estimates the relative expression of a gene of interest by comparing it to a reference gene, normalized to a control sample. The expression ratios were calculated as follows: 

$$\text{Ratio} = 2^{-\Delta\Delta Ct}$$

Assessment of genomic-DNA methylation and mRNA expression

Genomic DNA (gDNA) and total RNA from liver specimens were extracted with an RNA/DNA Extraction Kit (Qiagen), while a TRI-Reagent solution (Sigma) was used for both extractions from PBMC samples. DNA methylation rates in proximal AE2 promoter regions (upstream AE2a promoter and alternate AE2b1 and AE2b2 promoters within intron 2) were obtained at 1-base pair resolution by pyrosequencing. Briefly, gDNA aliquots (1 µg) were treated overnight with bisulphite (EpiTect-Bisulphite Kit from Qiagen) and used as template for PCR amplification with primers that match non-CpG regions within the bisulphite converted promoter sequences (see Table S2). 5’-biotinylated reverse primers allowed for resultant amplicons to be immobilized onto streptavidin-coated beads, followed by denaturation in 0.5 M NaOH, and sequencing of biotinylated reverse strands with respective forward primers (using a PyroMark™ Q96 pyrosequencer and PyroQ-CpG™ 1.0.9 software; Biotage-Qiagen).

The levels of mRNAs were assessed by real-time PCR in an iCycler iQ5 (BioRad), using reverse-transcribed total RNA and specific primers for AE2a, AE2b1 and AE2b2, and for GAPDH as normalizing control (Table S3). For calculations, we used the Livak/Schmittgen's method, though modified after estimating an average amplification efficiency of 80%, i.e. 1.8 ± ΔΔCt.

Lymphoblastoid-T2 cells

Human T cells (174xCEM.T2) – hybrid from B and T lymphoblasts – were cultured in RPMI with 25 mM Heps, 10% fetal bovine serum, and penicillin/streptomycin (Gibco-Invitrogen).

### Table 1. Main characteristics of the PBC-patient cohorts

|                        | Liver specimens, n = 20 (mean ± SD) | PBMC specimens, n = 16 (mean ± SD) |
|------------------------|-------------------------------------|------------------------------------|
| Sex (female:male)      | 18:2                                 | 15:1                               |
| Age (years)            | 54.3 ± 13.1                          | 52.5 ± 8.0                         |
| ALP (IU/L)             | 478.6 ± 430.7                        | 489.5 ± 308.4                      |
| GGT (IU/L)             | 238.0 ± 70.1                         | 1478.2 ± 252.4                     |
| ALT (IU/L)             | 51.2 ± 34.8                          | 46.0 ± 32.7                        |
| AST (IU/L)             | 45.4 ± 28.7                          | 40.6 ± 35.7                        |
| Bilirubin (mg/dl)      | 1.4 ± 2.2                            | 0.8 ± 0.4                          |

ALP, serum alkaline phosphatase; ALT, serum alanine aminotransferase; AST, serum aspartate aminotransferase; GGT, serum gamma glutamyltransferase; PBC, primary biliary cholangitis; PBMCs, peripheral blood mononuclear cells.

*The most relevant characteristics of the 2 different populations of patients with PBC from whom either liver samples or PBMCs were obtained, are indicated. The mean age of patients from whom normal liver samples were obtained (6 female and 8 male) was 58.2 years (SD: ± 12.9). The PBMC samples employed as normal controls were obtained from healthy donors after being completely anonymized.
Cells (1x10^5/well) were treated for 48 and 72 h with and without the demethylating agent 5-aza-2'-deoxycytidine (2 μM, Sigma) or the histone-deacetylase inhibitor trichostatin-A (300 nM, Sigma). Nucleic acids were extracted and analyzed as described above for the histone-deacetylase inhibitor trichostatin-A (300 nM; Sigma). Because the number of analyzed samples (10.78 ± 4.07% for PBC) was usually ~20 for each group, non-parametric tests were performed. Data are shown as mean ± SD unless otherwise indicated. *The most relevant characteristics (mean ± SD) of patients with OLDs from whom either liver samples or PBMCs were obtained, are indicated. Each OLD population was classified according to the etiology.

### Statistics
Data are shown as mean ± SD unless otherwise indicated. Statistical analyses were performed by using the GraphPad Prism-5 software (San Diego, CA). Because the number of analyzed samples was usually ~20 for each group, non-parametric tests were regularly employed (even though, according to D’Agostino-Pearson test, there were variables showing normally distributed values). Differences between PBC, OLD and normal control samples were analyzed with Kruskall-Wallis test followed by Mann-Whitney U test to analyze the differences between 2 groups. The Spearman’s rank-correlation coefficient \( r_s \) was also determined when pertinent. Two-tailed \( p \) values <0.05 were considered statistically significant. Receiver (or Relative) operating characteristic (ROC) curves were constructed and the area under ROC curves (AUC) was calculated to evaluate sensitivity and specificity of multiple CpG-dinucleotide sets within AE2 promoters. The criteria to obtain differentiating CpG-site combinations were ROC curves with \( p < 0.05 \) when PBC was compared with both NL and OLD livers, and ROC curves with \( p > 0.05 \) for comparisons between OLD and normal livers. The cut-off points on the ROC curves at which accuracy of PBC detection was maximal, were selected.

### Results

#### Cpg-methylation analysis of AE2 promoters in liver samples
Pyrosequencing of the proximal AE2a promoter region encompassing 18 CpG sites in liver gDNA revealed increased average methylation in PBC livers (24.01 ± 9.60%) compared to NL samples (10.78 ± 4.07%, \( p < 0.001 \)) and OLD specimens (13.19 ± 7.31%, \( p < 0.005 \)), while no significant differences were observed between OLD and NL samples (see also heat-map representations in Fig. 1). Because alternative transcriptional activity is normally prominent in the human liver, alternate AE2b2/ AE2b1 overlapping promoter regions were also analyzed in liver gDNA. Similarly to our observations for AE2a, pyrosequencing of 18 CpG sites in AE2b2 and 7 CpG sites in AE2b1 regions indicated that average methylation rates in PBC (65.71 ± 11.05% for AE2b1 and 49.14 ± 7.03% for AE2b2) were increased compared to NL samples (56.72 ± 8.99% for AE2b1, \( p < 0.05 \), and 38.55 ± 7.22% for AE2b2, \( p < 0.001 \)) and OLD specimens (56.66 ± 10.17% for AE2b1, \( p < 0.01 \), and 40.43 ± 7.38% for AE2b2, \( p < 0.001 \)), whereas no significant differences were observed between NL and OLD liver samples (see also heat maps in Fig. 1).

#### AE2 Cpg-methylation signatures in PBC livers
We then searched for minimal clusters of AE2 Cpg methylation that could specifically discriminate the PBC condition from both normal and OLD controls. We therefore analyzed average methylation rates of all possible combinations of assessed CpG sites, and performed ROC curve analysis of differentiation between PBC, NL and OLD livers. As illustrated in Fig. 2A-B, 2 minimal CpG clusters, one relative to the AE2a promoter (−274a, −254a, and −249a sites), and the other relative to the AE2b2 alternate promoter (−307b1, −280b1, −263b1, and −261b2 sites), gave highly significant ROC curves, with the highest fraction of PBC samples correctly identified as positive (i.e. highest sensitivity) and with the highest fraction of non-PBC samples correctly identified as negative (i.e. highest specificity). As expected, average methylation rates of these 2 clustered CpG sites were significantly higher in PBC than in normal and diseased controls (Fig. 2C-D). Noticeably, the hypermethylated CpG sites of the AE2b2 minimal cluster (−307b3, −280b3, −263b2, and −261b3 sites) are surrounding an HNF4 motif, closely upstream of the HNF1 site, in the AE2b2 alternate promoter (see Fig. 1, Table 2. Main characteristics of patients with OLDs.

### Patients with OLDs from whom liver specimens were obtained

| Patients (female/male) | Age (years) | ALP (IU/L) | GGT (IU/L) | ALT (IU/L) | AST (IU/L) | Bilirubin (mg/dl) |
|------------------------|-------------|------------|------------|------------|------------|------------------|
| ALD 0 / 13             | 56.1 ± 9.4  | 329.3 ± 190.7 | 103.1 ± 99.7 | 41.1 ± 19.0 | 56.2 ± 19.8 | 3.8 ± 1.9        |
| NAFLD 2 / 2            | 58.9 ± 11.7 | 275.8 ± 92.3  | 199.8 ± 61.2 | 117.3 ± 81.6 | 41.7 ± 14.6 | 0.7 ± 0.4        |
| TxH 0 / 1              | 70.7        | 1632.0      | 914.0      | 77.0       | 80.0       | 5.4              |
| VHC 0 / 3              | 59.7 ± 16.6 | 131.0 ± 30.1  | 33.3 ± 21.5 | 44.0 ± 19.3 | 22.7 ± 2.1  | 0.9 ± 0.5        |
| PSC 0 / 1              | 54.6        | 1598.0      | 309.0      | 52.0       | 48.0       | 5.6              |
| SBC 1 / 1              | 68.5 ± 4.5  | 574.5 ± 632.9 | 103.0 ± 108.9 | 12.0 ± 5.7 | 16.5 ± 3.5  | 1.0 ± 0.4        |

ALD, alcohol-related liver disease; ALP, serum alkaline phosphatase; ALT, serum alanine aminotransferase; AST, serum aspartate aminotransferase; GGT, serum gamma glutamyltransferase; NAFLD, non-alcoholic fatty liver disease; OLDs, other liver diseases; PBMCs, peripheral blood mononuclear cells; PSC, primary sclerosing cholangitis; SBC, secondary biliary cirrhosis; TxH, toxic hepatitis; VHC, viral hepatitis C.

*The most relevant characteristics (mean ± SD) of patients with OLDs from whom either liver samples or PBMCs were obtained, are indicated. Each OLD population was classified according to the etiology.
Additional ROC curve analysis combining the 2 minimal CpG clusters of AE2a and AE2b2 promoters resulted in the highest AUC (Fig. S1). ROC curve analyses, therefore, allowed us to differentiate the PBC condition from the controls (NL and OLD) with high sensitivity, specificity and accuracy. Altogether, our data support the notion that, in the liver, AE2 promoter
The female preponderance in PBC is remarkable; the ratio of affected females to males is as high as 10:1.43 Recently, methylation rates of PBC-associated minimal CpG clusters in (C) AE2a promoter region among patients were significantly lower in PBC livers than in NL samples (<0.001 for both AE2a and AE2b2 mRNAs and p < 0.05 for AE2b1 mRNA). Concerning the mRNA levels in our current OLD cohort with just OLD and NL samples (without PBC samples), they were markedly lower in the specimen subset from patients with advanced and severe cholestasis (n = 16, Table S1 and Fig. S2), whereas in samples from patients with moderate cholestasis (n = 8, Table S1) the mRNA levels were significantly higher (Fig. S2). In PBC specimens, however, the AE2 mRNA levels were always consistently diminished, regardless of whether patients had severe or moderate cholestasis (Fig. S2).

To assess whether diminished AE2 gene expression was associated with increased promoter methylation, we performed correlation analyses between the levels of AE2 mRNA isoforms and average CpG methylation rates. Significant negative correlations were obtained for the cohort including all liver samples, i.e. in the PBC+NL+OLD cohort (Table S5). Correlation coefficients were higher in the cohort including PBC livers and NL samples only (PBC+NL cohort, with no OLDs samples), while analysis of the cohort with just OLD and NL samples (without PBC samples) gave no significant correlations (Table S5). In fact, the methylation rates of AE2 promoter regions in the liver subset of severely cholestatic patients with OLDs were in the range of those in patients with OLDs and moderate cholestasis (Fig. S3), despite the encountered differences in AE2 mRNA levels between the two OLD subsets (Fig. S2). These data concur with the view that the diminished AE2 expression observed in the former OLD subset is unrelated to CpG methylation but due to severe cholestasis, while the diminished liver expression of AE2 mRNAs in PBC livers appears to be amply justified by the increased CpG methylation in AE2 promoter regions.

**AE2 CpG methylation and AE2 mRNA expression in PBMCs**

Pyrosequencing analysis of the AE2a promoter in PBMCs showed >2-fold increased average methylation in PBC (20.18 ± 8.83%) compared to healthy and diseased controls (HVs 7.16 ± 1.37% and OLDs 9.22 ± 3.04%; p < 0.01 each; see heat-map representations in Fig. 3A). ROC curve analysis of average methylation rates of all possible combinations of assessed CpG sites revealed a minimal cluster of 3 particular CpG-cytosines (at positions −329a, −299a, and −291a) that specifically differentiated PBC samples from both controls (Fig. 3B). Indeed, the average methylation rate of these combined CpG sites was significantly higher in PBC (26.0 ± 13.22) than in both healthy (7.75 ± 2.93; p < 0.001) and diseased controls (10.32 ± 7.48; p < 0.001; Fig. 3C).

Quantitative-PCR analysis of PBMCs from 12 patients with PBC, from 25 patients with OLDs and from 16 HVs showed consistent expression of AE2a mRNA in all PBMC samples, while alternative AE2b1 and AE2b2 mRNA variants could hardly be detected (not shown). In agreement with our previous findings,17 the levels of AE2a mRNA in PBMCs were significantly diminished in PBC compared to HV and OLD samples (p < 0.01 each; Fig. S4). Like liver tissue, negative correlations between AE2a mRNA levels and average methylation rates of AE2a promoter were observed in cohorts with PBC and control samples (Table S6).

Pyrosequencing of alternate AE2b2 and AE2b1 promoter regions in randomly selected PBMC gDNA samples from individuals with PBC and OLDs, and from HVs revealed consistent methylation (Fig. S5). To investigate whether methylation of alternate AE2b2/AE2b1 promoter regions might hinder the
expression of alternative AE2b2 and AE2b1 mRNAs in immune cells we cultured lymphoblastoid T2 cells with and without the demethylating agent 5-aza-2’-deoxycytidine and determined mRNA levels. Baseline, T2 cells were found to express AE2a mRNA but not alternative AE2 variants (Fig. 4), which concurred with the observations in PBMCs. Noticeably, T2 cells treated with 5-aza-2’-deoxycytidine showed substantial expression of alternative AE2b2 and AE2b1 mRNAs (Fig. 4), strongly suggesting that demethylation leads to upregulated transcription from alternate AE2b2/AE2b1 promoters. Moreover, 5-aza-2’-deoxycytidine

Fig. 4. CpG methylation is involved in the dormant expression of alternative AE2b1 and AE2b2 mRNAs in human lymphoblastoid T2 cells. Baseline, the overall methylation rates of AE2a promoter regions in T2 cells are the following: 8.50 ± 8.25% for the upstream AE2a region and 61.83 ± 26.11% and 94.14 ± 6.31% for the alternate AE2b2 and AE2b1 regions (respectively). The levels of alternative AE2b2 and AE2b1 mRNAs as well as those of the complete AE2a transcript were determined by real-time PCR in cultured T2 cells treated for 48 h with either 5-aza-2’-deoxycytidine, trichostatin-A, or just vehicle as control. Values (normalized for GAPDH mRNA) are given as fold expression relative to values of the control with vehicle alone. Significant p values refer to comparisons versus the control with vehicle. Significant p values (p < 0.05) were obtained by Kruskall-Wallis test followed by Mann-Whitney U test.
treatment was followed by a 3.6-fold increase in AE2a mRNA levels (Fig. 4), most probably due to complete release of the AE2a promoter from a baseline status of low-rate methylation (average methylation rate of 8.50 ± 8.25%).

Finally, we tested a possible role of histone acetylation-deacetylation for AE2 transcription and treated T2 cells with the HDAC inhibitor trichostatin-A during 48 and 72 h. In contrast to the effects observed with 5-aza-2’-deoxycytidine, no significant changes in the levels of mRNAs were found with trichostatin-A (see Fig. 4 depicting the results at 48 h).

Discussion

Here we report that significant epigenetic modifications of the AE2 gene occur in the liver and PBMCs of patients with PBC, which could explain the reduced gene expression in these patients. Thus, we observed an increased CpG-cytosine methylation of AE2a, AE2b1 and AE2b2 proximal promoters in PBC livers compared to normal and diseased controls. Hypermethylation affects more intensely particular CpG sites in each promoter region. ROC curve analysis found 2 minimal clusters of hypermethylated CpG-cytosines (1 comprised of 3 CpG-cytosines in the upstream AE2a promoter, and another comprised of 4 CpG sites in the alternate AE2b2 promoter, see Fig. 2), which could specifically differentiate PBC from both normal and OLD samples. Moreover, average methylation rates of combined CpG-cytosines were found to inversely correlate with the levels of AE2 mRNA driven by respective promoter regions in the liver.

Analogous data and correlations were observed in PBMCs but referred only to the upstream AE2a promoter and AE2a transcript. ROC curve analyses also found a minimal cluster composed by 3 CpG-cytosines in the AE2a promoter, the average methylation of which could specifically differentiate PBMC samples of patients with PBC from normal and OLD peripheral immune cells (Fig. 3). On the other hand, alternate AE2b2/AE2b1 promoter regions were ascertained to be silent not only in PBC but also in normal and diseased PBMCs. These regions show constitutive increased CpG-cytosine methylation that presumably prevents the expression of alternative AE2 transcripts. This view is strongly supported by our findings in lymphoblastoid T2 cells, in which dormancy of expression of AE2b2 and AE2b1 mRNAs could be activated when T2 cells were treated with the demethylating agent 5-aza-2’-deoxycytidine. Interestingly, such a treatment also increased the expression of AE2a mRNA, revealing that further release from basal low-rate methylation in the AE2a proximal promoter might result in amplified transcriptional activity. Treatment with trichostatin-A, however, had no effect on AE2 promoter activities in T2 cells, which suggests that histone deacetylation is poorly involved in dormant alternative expression of AE2 in these immune cells.

Altogether, our findings indicate that hypermethylation of the AE2 promoter regions might be an important mechanism leading to decreased expression of AE2 mRNAs in the liver and PBMCs of patients with PBC. It may be speculated that methylation of CpG-cytosines located in the vicinity of particular motifs might preclude the recruitment of DNA binding proteins and cause transcriptional inactivation.25,36 Interestingly, the minimal cluster of CpG-cytosines in the AE2a promoter that are hypermethylated in PBC livers spans near the equivalent minimal cluster detected in PBMCs from patients with PBC; for instance, most AE2a-CpG sites with increased methylation in liver and PBMC samples from patients with PBC locate close to Sp1 and ERG-1/WT1 motifs (Figs. 1 and 3). Consequently, the clusters detected in the AE2a promoter region (1 in liver samples and another in PBMCs from patients with PBC) can affect similar motifs in the promoter. It might also be speculated that methylated cytosines serve as docking sites for methyl-CpG-binding domain (MBD) proteins and repress transcription indirectly via recruitment of corepressors and chromatin modification.25,36

Transcription from alternate AE2b2 and AE2b1 overlapping promoter regions is quite restricted to the liver and kidney in humans, when compared to rodents.39 Most probably, liver restriction is connected to motifs for liver-enriched and related transcription factors like HNF1, CBP, and glucocorticoid receptor encountered in those overlapping promoter regions in the human gene.25,42 Of notice, most AE2b2/AE2b1-CpG sites characteristically hypermethylated in the liver of PBC patients locate in the neighborhood of sites and motifs for those liver-related transcription factors.39 In fact, the minimal cluster of hypermethylated CpG-cytosines in the AE2b2 alternate promoter which discriminates PBC livers from normal liver samples and OLD samples spans a region encompassing an HNF1 site25,42 and an upstream HNF4 motif.39

In the liver, AE2 is involved in biliary bicarbonate secretion.20,27 This exchanger protein is therefore directly responsible for creating the “bicarbonate umbrella” along the biliary tree that protects cholangiocytes from the proapoptotic effects of bile salts by maintaining them deprotonated.45 In a context of reduced liver AE2 expression and AE2 deficiency, there may occur defective bicarbonate umbrella and subsequent entry of protonated bile salts into cholangiocytes (concisely and nicely reviewed in ref. 45). Entry of highly hydrophobic bile salts can promote reactive oxygen species (ROS) production, and lead to enhanced inflammatory cytokine and chemokine responses.46 ROS production might also contribute to further decreasing AE2 expression.46 On the other hand, AE2 knockdown in vivo was shown to result in increased ROS production in the liver.46 Additionally, AE2 knockdown experiments in cholangiocytes led to intracellular accumulation of bicarbonate and increased expression and activity of soluble adenylyl cyclase (sAC).47 This bicarbonate sensor was found to sensitize cholangiocytes to bile salt-induced apoptosis through the intrinsic apoptotic pathway.47 Interestingly, cholangiocytes were previously reported to translocate immunologically intact PDC-E2 to apoptotic bodies and create an apotope,48 that may favor the development of AMAs. The triad of bile-duct cell derived apoptopes, macrophages from patients with PBC, and AMAs were also found to trigger an intense production of proinflammatory cytokines.49 More recently, cultured human cholangiocytes treated with proinflammatory cytokines typically overexpressed in PBC livers (such as IL8, IL12, IL17, IL18, and TNF-α) were shown to enhance the expression of miR-506, that induced PBC-like features in these bile-duct cells and promoted immune activation.50 Noticeably, bile-duct cells of PBC livers were described to upregulate miR-506, which can inhibit translation of AE2 transcripts and contribute to further decreasing the expression of AE2 protein.44

Previously reported experiments in animal models showing that highly hydrophobic bile salts might decrease AE2 mRNA expression through ROS production46 seemingly have a correlate in the subset of our current diseased liver control specimens from severely cholestatic patients (Fig. S2). In this subset of diseased control samples, we found markedly low levels of AE2 mRNAs despite AE2 promoter methylation being comparable to that in normal-liver control samples (as well as to those in diseased control samples from patients with OLDs and moderate cholestasis; Fig. S3). In PBC livers, however, promoter methylation was significantly increased (Fig. S3) and AE2 mRNA levels were consistently diminished (Fig. S2), regardless of whether patients had severe or moderate cholestasis.
Overall, these data support the notion that diminished AE2 gene expression in PBC livers primarily results from increased AE2 CpG methylation, whereas in OLD specimens from severely cholestatic patients, the diminished liver expression of AE2 gene occurs as a direct consequence of the elevated levels of hydrophobic bile salts, unrelated to CpG methylation. Our earlier findings in Ae2-knockout mice, as well as the aforementioned AE2 knockout experiments (both in vivo and in bile-duct cells), clearly show that a primary failure in AE2 gene expression may result in detrimental cascades in the liver.

In PBMCs, AE2 is involved in intracellular pH regulation and immunological homeostasis. Thus AE2 promoter hypermethylation and deficient AE2 mRNA expression in patients with PBC may contribute to the immune dysregulation PBC patients typically have. Indeed the particular predisposition to activation of immune cells against bile-duct cells in PBC resembles the dysfunctions of T cells observed in our Ae2-knockout animal model, in which they interact in the liver with highly immunogenic AE2-deficient bile-duct cells. In contrast to PBC, no autoimmune cholangitis is expected to develop in severely cholestatic patients with OLDs despite dramatically decreased AE2 mRNA levels in the liver, since PBMCs do not appear to be equally affected by the severe cholestasis. Thus, the idiosyncratic conjugation of anomalies putatively streaming from AE2 hypermethylation in both the liver and PBMCs in patients with PBC may constitute a crucial 2-arms prerequisite for the development of autoimmune cholangitis (Fig. S6), and provide relevant clues to unravel the enigmatic pathogenesis of the disease.

A series of epigenetic alterations on the X chromosome and autosomal chromosomes were previously reported in PBC. Some genes appear hypermethylated while other genes are with decreased DNA methylation, indicating that hypermethylation is not a widespread phenomenon in PBC. Therefore, the increased methylation of the AE2 gene currently observed in our study in PBC samples might be viewed as disease specific in the context of additional epigenetic modifications. Disclosing the cause(s) of such modifications in PBC should be a priority for the related studies in the near future. Certainly, PBC is a multifactorial liver disease that may ensue from highly complex interactions between genetic and environmental factors, as stressed by GWAS data and more conventional genetic and epidemiological studies. Environmental exposures may result in epigenetic modifications, particularly DNA methylation. Smoking, for instance, which has been associated with high risk of PBC, was also related with increased DNA-methyltransferase expression. Therefore, DNA methylation affecting AE2 and additional genes, may constitute a link between genetic and environmental risk factors in PBC pathogenesis.

In summary, AE2 deficiency in patients with PBC may render cholangiocytes more immunogenic and susceptible to autoimmune attack, whereas an equivalent defect in PBMCs may alter immunological homeostasis. Additional identified (and not yet identified) genetic and epigenetic modifications in genes pertinent to immunity may further boost a pivotal contribution of AE2 deficiency, and lead to immune dysregulation and autoimmunity (Fig. S6).

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Declaration of Competing Interest
The authors declare no conflicts of interest that pertain to this work.

Authors’ contributions
JFM made the conception and design of the study. FA, JP and AP also contributed to the interpretation of the data. FA, JFM and AP performed the statistical analysis. JFM, FA, JP and AP contributed to the manuscript. JFM, JP and AP were responsible for funding acquisition.

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Supplementary data
Supplementary data associated with this article can be found in the online version, at https://doi.org/10.1016/j.jhepr.2019.05.006.

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