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Promoter hypermethylation of the *AE2/SLC4A2* gene in PBC

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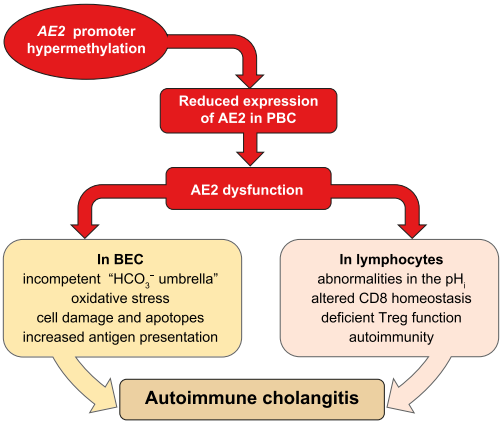
Graphical abstract



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^-/HCO_3^- 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.

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.[2–5] 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.[6] Genome-wide-association studies (GWAS)[7–10] and dense fine-mapping association studies[11] 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 choleresis, we have been postulating 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.[19]

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^-/HCO_3^- anion exchange (AE), which can be stimulated by cAMP, the second messenger for secretin signal transduction.[22] *AE2/SLC4A2* gene-silencing experiments indicated that AE2 is the main carrier for this activity in cholangiocytes.[25–27] Previously, we reported that bile-duct cells isolated from PBC patients exhibit defective cAMP-stimulated AE activity,[20] and that PBC livers have diminished AE2 expression at the luminal membrane of the biliary epithelium.[18] Also, we reported that *AE2* mRNA levels are decreased in liver biopsies and peripheral blood mononuclear

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cells (PBMCs) from patients with PBC.[17] 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.[28–30]

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.[31] 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.[32] Also, a synonymous variation in exon 6 (rs2303932) was related with the progression of the disease in a French population.[33] More recent findings provided evidence for upregulation of microRNA 506 in PBC bile-duct cells being involved in the decreased liver expression of AE2 protein through blocking the translation (but not the transcription) of AE2 messages.[34] However, the genetic and/or epigenetic factors responsible for the decreased *AE2* mRNA expression in PBC[17] remain to be fully elucidated. Since hypermethylation of CpG-cytosines in promoter regions is a frequent mechanism for transcriptional inactivation,[35–37] we explored these possible variations in *AE2/SLC4A2* promoter regions. Thus, we analyzed the methylation rates of proximal CpG-cytosines in the widely expressed upstream promoter *AE2a* and in the tissue-restricted alternate overlapping *AE2b2/AE2b1* promoter regions (located within intron 2 of the *AE2/SLC4A2* gene),[38,39] in liver and PBMC samples from patients with PBC and normal and diseased controls. Additionally, we determined the correlation between methylation rates in *AE2* promoters and the levels of respective *AE2* mRNAs in these samples.

Patients and methods

Human samples and patients

Liver specimens (n = 20) and PBMCs (n = 16) from patients with PBC were obtained at the Clinic University of Navarra (Pamplona) and the Hospital Clinic (Barcelona) before patients started their treatment with UDCA. Liver specimens were frozen samples of either diagnostic liver biopsies or pieces of liver explants from patients transplanted because of advanced PBC. Diagnosis of PBC was based on biochemical cholestasis, AMA positivity (as determined by immunofluorescence), and compatible histology – presymptomatic forms with normal alkaline phosphatase were also considered – (see Table 1 for patient characteristics and biochemical parameters). Pieces of liver explants from patients transplanted because of other liver diseases (OLDs) (n = 24) and PBMCs from additional patients with OLDs (n = 25) were included in the study as non-PBC diseased controls (see Table 2 for patient characteristics and biochemical parameters). The 24 patients with OLDs from whom liver explants were obtained were further split into patients with either moderate or severe cholestasis according to biochemical parameters, as detailed in Table S1. Additionally, normal bordering tissue samples (n = 14) obtained from surgery dissection of metastatic tumors in the liver were included as normal liver controls (NL). PBMCs used as normal controls (n = 16) were anonymized samples obtained from healthy blood-donors/volunteers (HVs). All patients gave written informed consent for the study, which was approved by the institutional Ethics Committees, in compliance with the ethical principles of the Declaration of Helsinki.

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)[38,39] 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,[40] though modified after estimating an average amplification efficiency of 80%, *i.e.* $1.8^{-\Delta\Delta CT}$.

Lymphoblastoid-T2 cells

Human T2 cells (174xCEM.T2) – hybrid from B and T lymphoblasts – were cultured in RPMI with 25 mM Hepes, 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 ± 270.1	147.8 ± 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 volunteers/blood-donors after being completely anonymized.

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
Patients with OLDs from whom PBMC specimens were obtained							
	Patients (female/male)	Age (years)	ALP (IU/L)	GGT (IU/L)	ALT (IU/L)	AST (IU/L)	Bilirubin (mg/dl)
ALD	1 / 10	57.6 ± 5.9	202.6 ± 59.2	31.4 ± 14.7	16.2 ± 10.4	28.5 ± 9.7	5.6 ± 4.3
NAFLD	0 / 1	46.0	109.0	38.0	70.0	28.0	1.4
TxH	1 / 0	45.0	1321.0	815.0	71.0	76.0	5.1
VHC	5 / 5	57.5 ± 13.8	124.9 ± 33.6	24.7 ± 18.0	61.7 ± 45.8	44.1 ± 34.0	0.6 ± 0.3
PSC	0 / 1	55.0	184.0	39.0	10.0	24.0	4.5
SBC	0 / 1	59.0	133.5	27.0	9.5	22.5	4.1

ALD, alcohol-related liver disease; ALP, serum alkaline phosphatase; ALT, serum alanine aminotransferase; AST, serum aspartate aminotransferase; GGT, serum gamma glutamyl-transferase; 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 ethology.

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 PBMCs.

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[41] in Fig. 1). Because alternative transcriptional activity is normally prominent in the human liver,[39] 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 ($-307b_2$, $-280b_2$, $-263b_2$, and $-261b_2$ 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 ($-307b_2$, $-280b_2$, $-263b_2$, and $-261b_2$ sites) are surrounding an HNF4 motif,[39] closely upstream of the HNF1 site,[25,42] in the *AE2b2* alternate promoter (see Fig. 1,

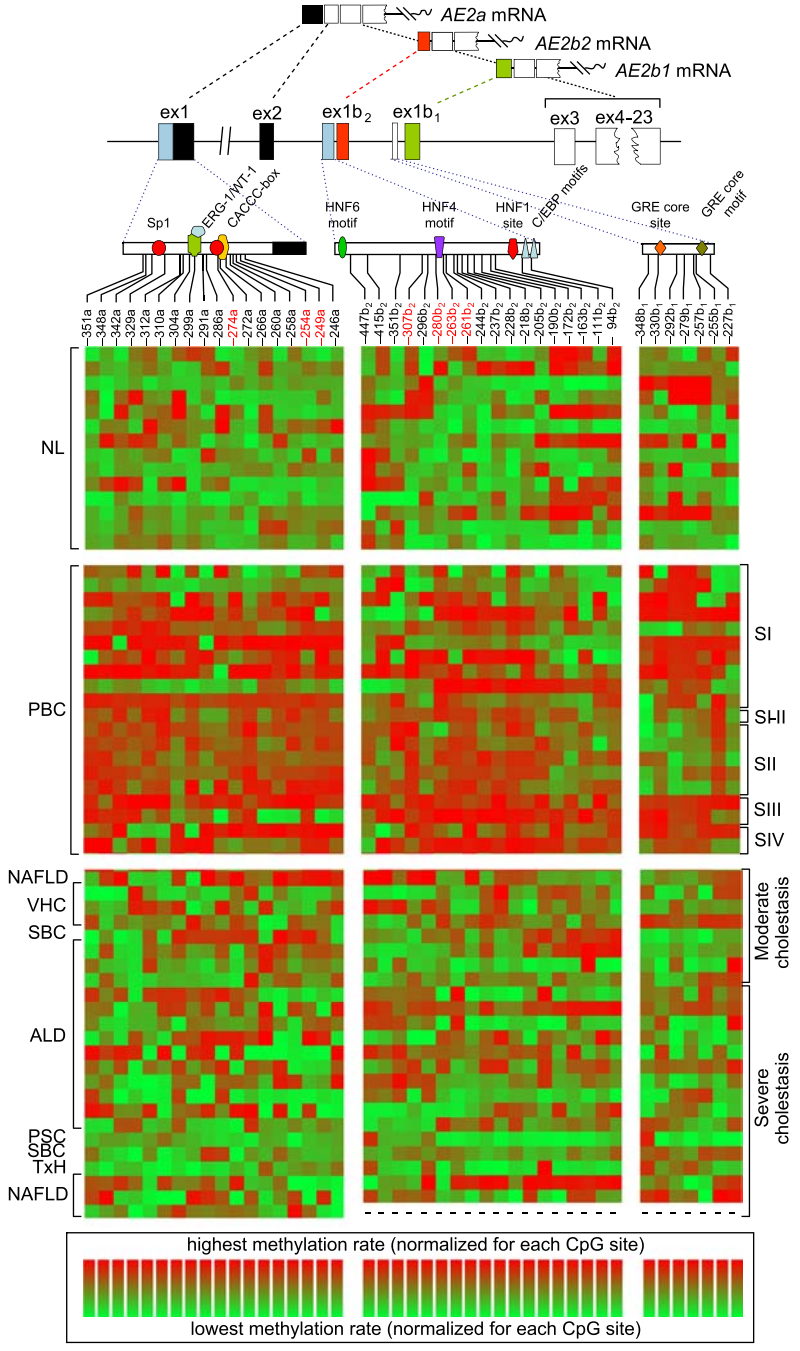

Fig. 1. Heat maps of CpG-methylation rates for *AE2* promoter regions in the liver. The methylation rates of CpG-cytosines within interrogated proximal regions of the upstream *AE2a* promoter (155-bp long; left panels), and alternate *AE2b2* (477-bp; middle *panels*) and *AE2b1* promoters (205-bp; right) were determined through pyrosequencing of bisulphite-converted gDNA from NL specimens (n = 14), PBC-liver samples (n = 20, grouped by disease stages SI-IV), and liver samples from patients with OLDs (n = 24). The upper diagrams show *AE2* promoter regions with relevant sites and putative motifs for transcription factors, the initial exons and the transcribed variants. Indicated CpG-dinucleotide positions, *i.e.* the negative numbers followed by either a, b_1 or b_2, are referred to cytosine locations upstream to respective +1 position, *i.e.*, A in ATG start codon in exon 2 for *AE2a* transcript, and in exons $1b_1$ and $1b_2$, for *AE2b1* and *AE2b2* mRNA variants, respectively. Red-labeled numbers indicate hypermethylated CpG sites conforming the PBC-associated methylation signatures of minimal clusters. Maps were obtained with the online Genesis software (http://genome.tugraz.at/serverclient/serverclient_download.shtml, originally designed at Graz University of Technology for microarray data; cf. ref.[41]). Here, the highest and lowest methylation-rate values – among all values obtained for every CpG site – were normalized in such a way that they ranged from 100% (*light red*) to 0% (*light green*) for each site (in accordance with respective color bars below). ALD, alcohol-related liver disease; bp, base pair; gDNA, genomic DNA; NAFLD, non-alcoholic fatty liver disease; NL, normal liver; OLDs, other liver diseases; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis; SBC, secondary biliary cirrhosis; TxH, toxic hepatitis; VHC, viral hepatitis C.

upper diagram). 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

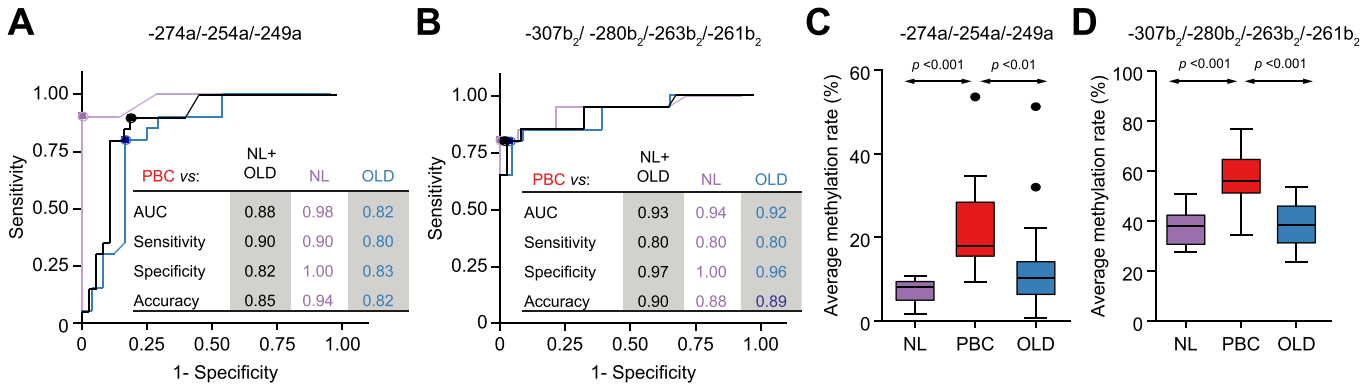

Fig. 2. PBC-associated methylation signatures of minimal CpG-site clusters differentiating PBC from NL and OLD liver samples. Highly significant ROC curves were obtained for average methylation of (A) a minimal CpG cluster within the *AE2a* promoter region (–274a, –254a, and –249a sites), and (B) another minimal cluster within the *AE2b2* alternate promoter (–307b$_2$, –280b$_2$, –263b$_2$, and –261b$_2$ sites); both ROC curve analyses also included comparisons of PBC versus NL +OLD cohort (non-PBC samples). Analyses always resulted in the highest fraction of PBC samples correctly identified as positive and with the highest fraction of non-PBC samples correctly identified as negative (highest sensitivity and highest specificity, respectively). Box plots to the right (each with minimum value, the first quartile, the median, the third quartile, and the maximum value) graphically depict the comparisons, between PBC and control liver samples, of average methylation rates of PBC-associated minimal CpG clusters in (C) AE2a promoter (–274a, –254a, and –249a sites), and in (D) *AE2b2* alternate promoter (–307b$_2$, –280b$_2$, –263b$_2$, and –261b$_2$ sites). Significant *p* values (<0.05) were obtained by Kruskall-Wallis test followed by Mann-Whitney *U* test. NL, normal liver; PBC, primary biliary cholangitis; OLD, other liver disease.

regions possess hypermethylation signatures of clustered CpG sites which are able to distinguish PBC from both NL and OLD.

Gender and *AE2* promoter hypermethylation

The female preponderance in PBC is remarkable – worldwide, the ratio of affected females to males is as high as 10:1.[43] Recently, DNA-methylation sex differences were reported for a series of genes and intergenic regions in the human liver.[44] We therefore decided to compare average methylation rates of the PBC-associated CpG clusters in NL from females with those from males, and found no differences, while highly significant differences were observed among females between NL and PBC (Table S4). This indicates gender is not a determinant factor for the hypermethylation changes detected in PBC specimens.

Negative correlations between mRNA levels of AE2 isoforms and methylation rates within respective promoter regions in liver samples

qPCR analysis of the 3 *AE2* mRNA isoforms showed their levels were significantly lower in PBC livers than in NL samples (p <0.001 for both *AE2a* and *AE2b2* mRNAs and p <0.05 for *AE2b1* mRNA). Concerning the mRNA levels in our current OLD samples (n = 24), 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 2 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

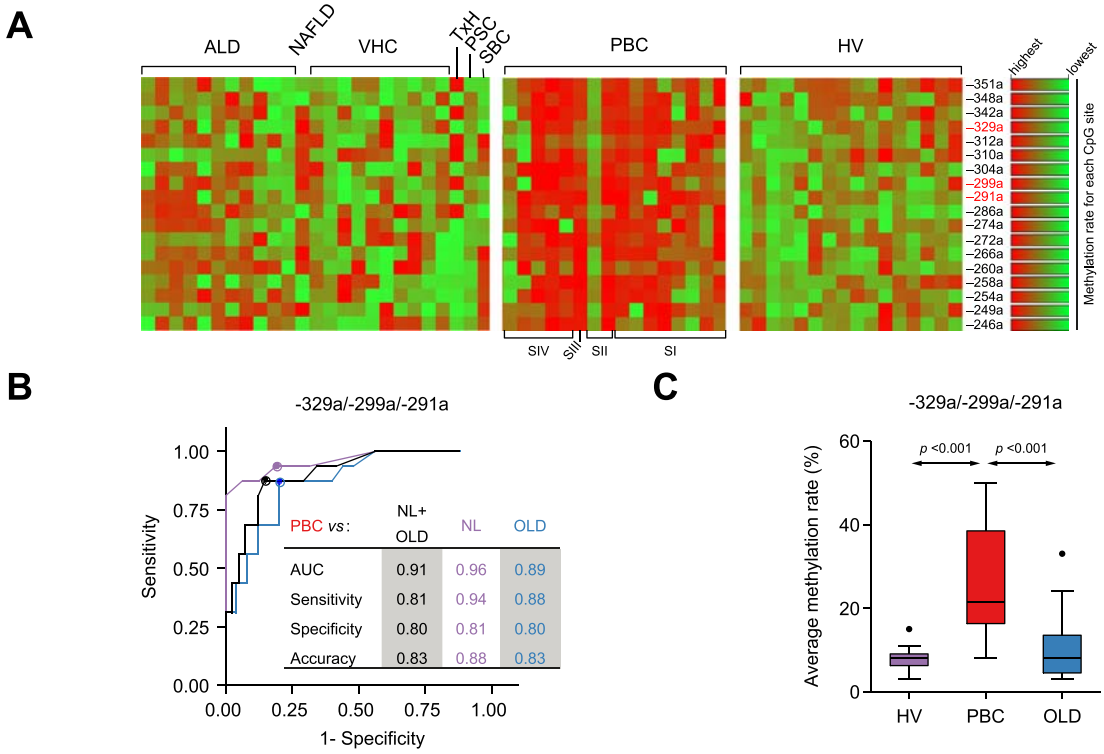

Fig. 3. PBC-associated methylation pattern of *AE2a* promoter region in PBMCs. (A) Heat map of methylation rates of CpG sites within the interrogated *AE2a* promoter in PBMCs from HVs (n = 16), patients with PBC (n = 16) and patients with OLDs (n = 25). Maps were obtained and normalized as described in Fig. 1 for the *AE2* promoter regions in the liver; here, normalization color bars locate to the right. (B) Highly significant ROC curves obtained for a minimal cluster of methylated CpG sites within *AE2a* promoter in PBMCs (–329a, –299a, and –291a), comparing PBC versus HV, OLD, and HV+OLD (*i.e.* non-PBC samples). (C) Comparisons of average methylation rates of the PBC-associated minimal CpG cluster within *AE2a* promoter in PBMCs (–329a, –299a, and –291a), between PBC and normal and diseased control samples. Significant p values (p <0.05) were obtained by Kruskall-Wallis test followed by Mann-Whitney *U* test. ALD, alcohol-related liver disease; HVs, healthy blood-donors/volunteers; gDNA, genomic DNA; NAFLD, non-alcoholic fatty liver disease; OLDs, other liver diseases; PBC, primary biliary cholangitis; PBMCs, peripheral blood mononuclear cells; PSC, primary sclerosing cholangitis; SBC, secondary biliary cirrhosis; TxH, toxic hepatitis; VHC, viral hepatitis C.

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

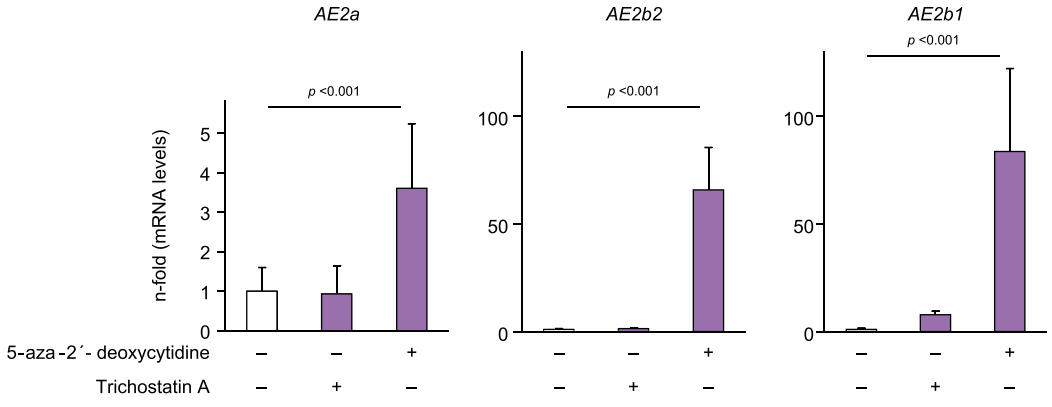

Fig. 4. CpG methylation is involved in the dormant expression of alternative *AE2b1* and *AE2b2* mRNAs in human lymphoblastoid T2 cells. Baseline, the overall methylation rates of *AE2* promoter regions in T2 cells are the following: 8.50 ± 8.25% for the upstream *AE2a* region and 61.83 ± 26.11% and 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* mRNAs 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 dormant 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.[35,36] 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.[35,36]

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 site[25,42] and an upstream HNF4 motif.[39]

In the liver, AE2 is involved in biliary bicarbonate secretion.[26,27] 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.[12]). 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 apotopes, 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.[34]

Previously reported experiments in animal models showing that highly hydrophobic bile salts might decrease *AE2* mRNA expression through ROS production[46] 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.

Altogether, 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,[28] as well as the aforementioned *AE2* knockdown experiments (both *in vivo*[46] and in bile-duct cells[47]), 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.[24,29,30] 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.[28–30] 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 conjunction 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.[51–53] 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.[6,54–56] Environmental exposures may result in epigenetic modifications, particularly DNA methylation.[57] Smoking, for instance, which has been associated with high risk of PBC,[56,58] was also related with increased DNA-methyltransferase expression.[59] 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.[29,30] 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.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions
JFM made the conception and design of the study. FA, JP and AP also contributed to the study design. FA, IH, ES and SM collected the data. FA, JFM and AP performed the statistical analysis. JFM, FA, JP and AP contributed to the interpretation of the data. FA, JFM and AP drafted and edited 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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