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

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Table S1. Main characteristics of OLD patients with either moderate or severe cholestasis, from whom liver specimens were obtained*

Patients with OLDs and moderate cholestasis

	Subjects (female / male)	Age (years)	ALP (x ULN)	GGT (x ULN)	ALT (x ULN)	AST (x ULN)	Bilirubin (x ULN)
ALD	0 / 3	56.2 ± 7.3	1.2 ± 0.02	0.6 ± 0.2	1.5 ± 1.0	1.6 ± 0.7	3.5 ± 3.0
NAFLD	1 / 0	54.4	2.0	5.1	1.7	1.4	0.5
VHC	0 / 3	59.7 ± 16.6	0.6 ± 0.2	0.9 ± 0.6	1.5 ± 0.7	0.9 ± 0.1	0.8 ± 0.5
SBC	1 / 0	65.0	0.6	1.0	0.7	0.9	0.7

Patients with OLDs and severe cholestasis

	Subjects (female / male)	Age (years)	ALP (x ULN)	GGT (x ULN)	ALT (x ULN)	AST (x ULN)	Bilirubin (x ULN)
ALD	0 / 10	55.3 ± 10.6	1.8 ± 1.0	6.5 ± 9.1	1.4 ± 0.6	2.6 ± 0.7	6.3 ± 8.2
NAFLD	1 / 2	60.4 ± 13.9	1.1 ± 0.02	7.2 ± 3.4	5.1 ± 5.3	5.2 ± 2.3	0.7 ± 0.4
TxH	0 / 1	70.7	7.9	24.1	2.7	3.2	4.9
PSC	0 / 1	54.6	7.7	8.1	1.8	1.9	5.1
SBC	0 / 1	72	4.9	4.7	0.3	0.6	1.2

ALD, alcoholic liver disease; ALP, serum alkaline phosphatase; ALT, serum alanine amino-transferase; AST, serum aspartate amino-transferase; GGT, gamma glutamyl-transpeptidase; NAFLD, non-alcoholic fatty liver disease; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis; and SBC, secondary biliary cirrhosis; TxH, toxic hepatitis; VHC, viral hepatitis C.

*The biochemical criteria to include patients in the OLD cohort with severe cholestasis were serum levels higher than 2.5 times the upper limit of normal (ULN, mean ± SD) of two or more cholestatic markers –ALP, GGT, and total bilirubin–, or only one of these accompanied by >2.5x ULN of ALT and/or AST; remaining patients were included in the OLD cohort with moderate cholestasis. The criteria were independent of the disease etiology. The sex-dependent limits of normality were given by the biochemistry service of the Clinic University of Navarra.

Table S2. Oligonucleotides used for PCR amplification and pyrosequencing of *AE2* promoter regions in bisulphite-treated genomic DNA

Promoter*	Forward	Reverse	Amplicon size	Flanking positions
AE2a	GAGTGGAGAAGTTTGGGGTAGA	*Biotin*–ACTTCTCCCAACTCAACTTTCC	155 bp	–395a / –241a
AE2b1	AGGGGAGTTAGGAGATGGAT	*Biotin*–AATATACAACCCCCTCCATAAC	205 bp	–409b_1 / –205b_1
AE2b2-upstream	CCCTAAATCTCTCACCCAAC	*Biotin*–GGTAGGGGTGAATTTGAGAA	477 bp	–481b_2 / –5b_2
AE2b2-downstream	TACTACCATCCCTACCCTAACT	*Biotin*–GGAAAATTTAAAGGGGGTTATGT		

*Promoter amplicons were pyrosequenced with respective forward primers; each sequencing primer could read around 100-110 bp. Flanking positions (numbers followed by either a, b_1 or b_2) are referred to the locations of the 5' nucleotide of forward and reverse primers; they are negative digits to indicate positions upstream of respective +1 positions (A in ATG start codons in exon 2 for *AE2a* transcript, in exon 1b_1 for *AE2b1* variant, and in exon 1b_2 for *AE2b2* transcript).

Table S3. Oligonucleotides used for real-time PCR to determine the levels of *AE2* mRNAs

mRNA	Forward	Reverse*	Amplicon size	Flanking positions
AE2a	TCCAGAGCGAGCGGGTTATG	GAGGACTGGCGGTGGTACTCAAAGTC	310 bp	–77a to +233a
AE2b1	CGCCCGCAGGATGACTCA	GAGGACTGGCGGTGGTACTCAAAGTC	201 bp	$-10b_1$ to $+191b_1$
AE2b2	CCCTCCTTCTCAGGTTCACCCCTGCC	GAGGACTGGCGGTGGTACTCAAAGTC	236 bp	$-30b_2$ to $+206b_2$
GAPDH	CCAAGGTCATCCATGACAAC	TGTCATACCAGGAAATGAGC	465 bp	+482 / +946

*As reverse primer for the 3 *AE2* mRNA isoforms, an identical oligonucleotide encompassing sequences of common exons 3 and 4 was used. Flanking positions (numbers followed by either a, b_1 or b_2) are referred to the locations of the 5' nucleotide of forward and reverse primers; positions upstream and downstream of respective +1 positions (A in ATG start codons in exon 2 for *AE2a* transcript, in exon $1b_1$ for *AE2b1* mRNA variant, and in exon $1b_2$ for *AE2b2* variant) are numbered as negative and positive digits, respectively. Sequences of primer pairs for the normalizing message GAPDH were both downstream of the start codon.

Table S4. Gender and methylation rates of PBC-associated minimal CpG clusters in the liver*

	Average methylation rates of CpG clusters		
	–274a, –254a, –249a	**–307b$_2$, –280b$_2$, –263b$_2$, –261b$_2$**	**Combined cluster**
Female NL (n = 6)	8.1 ± 2.8	39.4 ± 9.2	23.7 ± 3.9
Male NL (n = 8)	6.3 ± 2.8	36.8 ± 6.7	21.6 ± 3.9
Female PBC (n = 18)	21.3 ± 10.7	58.2 ± 9.8	39.7 ± 7.0
Comparisons			
Female NL *versus* Male NL	ns (p = 0.266)	ns (p = 0.572)	ns (p = 0.362)
Female NL *versus* Female PBC	***p* <0.0001**	***p* = 0.0003**	***p* <0.0001**

*Average methylation rates of PBC-associated minimal CpG clusters in normal liver samples from female individuals (Female NL) were compared with those in normal liver samples from males (Male NL) and with those in liver samples from female PBC patients (Female PBC).

Table S5. Significant correlations between methylation rates of CpG sites within *AE2* promoters and *AE2* mRNA levels in the liver

Average CpG methylation*	*versus*	mRNA levels			
		PBC+NL			PBC+NL+OLD
		***AE2a* mRNA**	***AE2b2* mRNA**	***AE2b1* mRNA**	***AE2a* mRNA**
		r_S/ ***p* value**	r_S/ ***p* value**	r_S/ ***p* value**	r_S/ ***p* value**
18 *AE2a*-CpG sites		–0.53 / 0.0012	–0.56 / 0.0006	–0.46 / 0.0069	–0.28 / 0.0371
–274a; –254a; –249a		–0.62 / <.0001	–0.66 / <.0001	–0.48 / 0.0038	–0.32 / 0.0147
18 *AE2b2*-CpG sites			–0.36 / 0.0388	–0.37 / 0.0326	
–307b2; –280b2; –263b2; –261b2		–0.38 / 0.0278	–0.45 / 0.0076		
–274a; –254a; –249a; –307b2; –280b2; –263b2; –261b2		–0.44 / 0.0095	–0.54 / 0.0009	–0.47 / 0.0049	

NL, normal liver; OLDs, other liver diseases; PBC, primary biliary cholangitis; PBMCs, peripheral blood mononuclear cells.

*Average methylation either considers the 18 CpG sites analyzed in *AE2a* promoter region, the 18 CpG sites within *AE2b2* promoter, the 2 PBC-associated minimal CpG clusters (–274a, –254a, and –249a in *AE2a* promoter and –307b2, –280b2, –263b2 and –261b2 in *AE2b2* promoter), or the combination of both clusters.
r_S, Spearman's rank-correlation coefficient; only significant correlations are shown.

Table S6. Significant correlations between average CpG methylation rates within *AE2a* promoter and *AE2a* mRNA levels in PBMCs

Average CpG methylation*	*versus*	*AE2a* mRNA levels	
		PBC+HV cohort r_S/ *p* value	PBC+HV+OLD cohort r_S/ *p* value
18 *AE2a*-CpG sites		–0.43 / 0.0233	–0.29 / 0.0361
–329a, –299a & –291a		–0.42 / 0.0258	

HVs, healthy blood-donors/volunteers; OLDs, other liver diseases; PBC, primary biliary cholangitis; PBMCs, peripheral blood mononuclear cells.

*Average methylation considers the 18 CpG sites analyzed in the *AE2a* promoter or just the 3 CpG sites of the PBC-associated minimal cluster within this same promoter.
r_S, Spearman's rank-correlation coefficient; only significant correlations are shown.

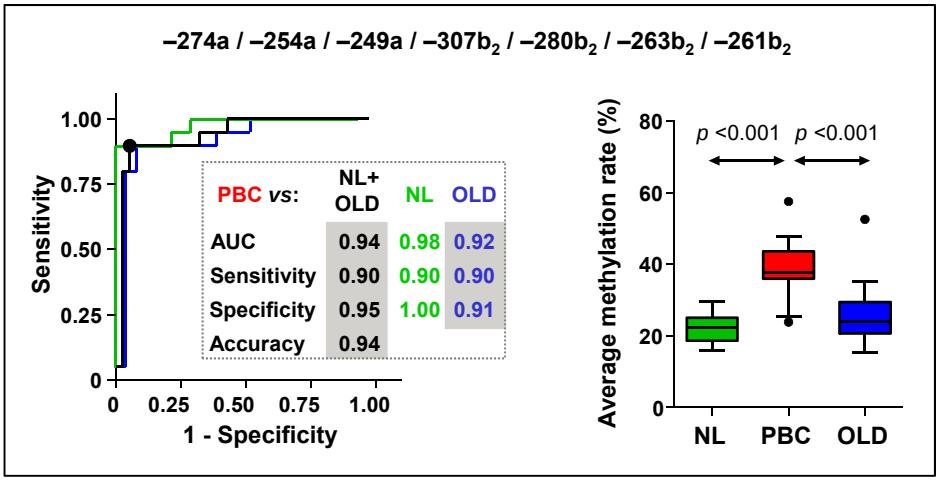

Fig. S1. Combination of the two minimal clusters of methylated CpG sites within *AE2a* and *AE2b2* promoters in the liver gives rise to the most PBC-specific methylation pattern. Left panel, highly significant ROC curves were obtained for the combined cluster of methylated CpG sites within *AE2* promoter regions in liver samples (–274a, –254a, –249a, –307b_2, –280b_2, –263b_2, and –261b_2 sites), when comparing PBC versus NL, OLD, and NL+OLD (i.e. non-PBC samples). Box plots to the right (each with minimum value, first quartile, median, third quartile, and maximum value), graphically depict the comparisons of average methylation rates of the combined PBC-associated CpG cluster within *AE2* promoter regions (–274a, –254a, –249a, –307b_2, –280b_2, –263b_2, and –261b_2 sites), between PBC and normal and diseased control liver samples. 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.

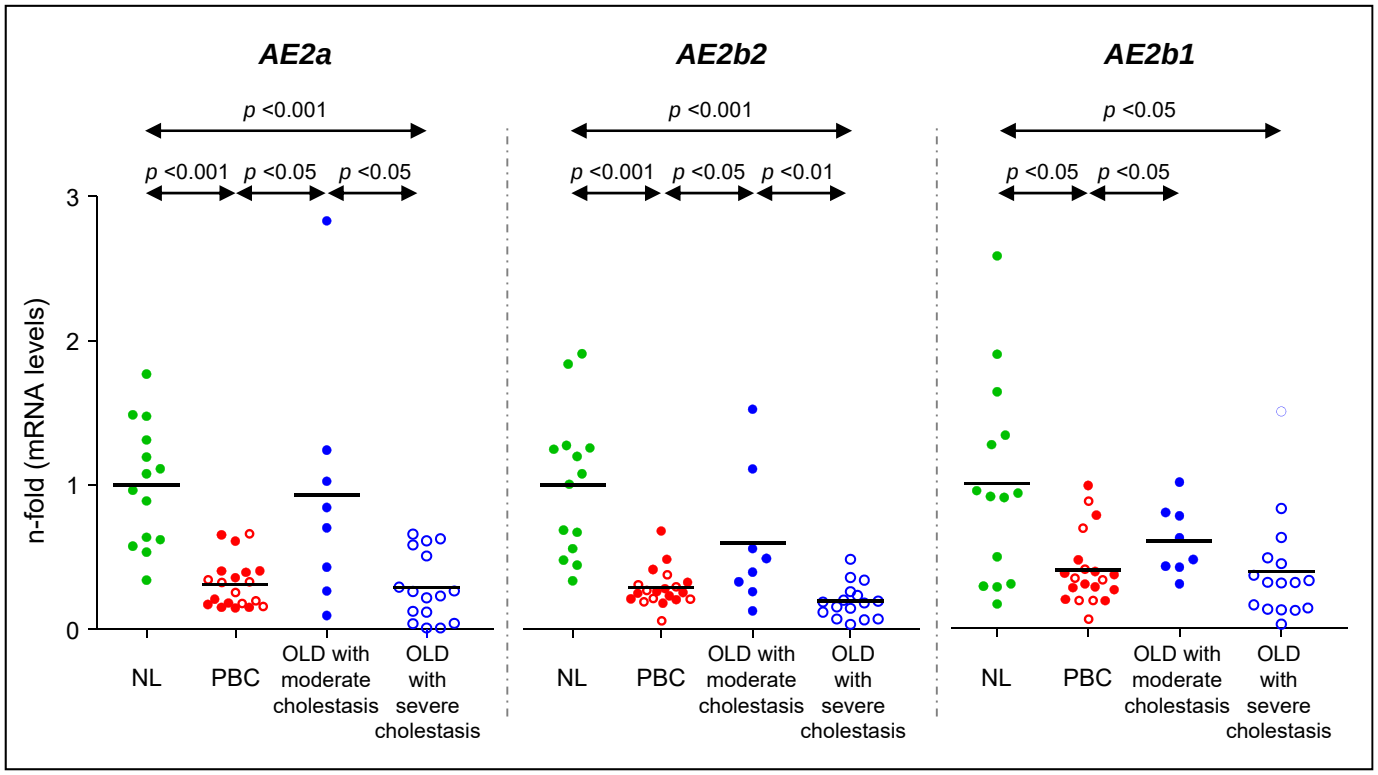

Fig. S2. Expression of *AE2* mRNA isoforms in liver samples. The levels of *AE2a*, *AE2b2* and *AE2b1* mRNA variants were determined by real-time PCR in liver samples from patients with PBC (n=20) and from patients with OLDs (n = 24), and in normal liver (NL) tissue specimens (n = 14). OLD samples were sub-grouped as diseased controls from patients with moderate (n = 8) or severe (n = 16) cholestasis (cf. Table S1). Values (normalized for GAPDH mRNA) are given as fold expression relative to mean value in NL for the respective AE2 isoform. Significant *p* values (<0.05) were obtained by Kruskall-Wallis test followed by Mann-Whitney *U*-test. In PBC and OLD: with moderate (closed circles) or severe (open circles) cholestasis. PBC, primary biliary cholangitis; OLDs, other liver diseases.

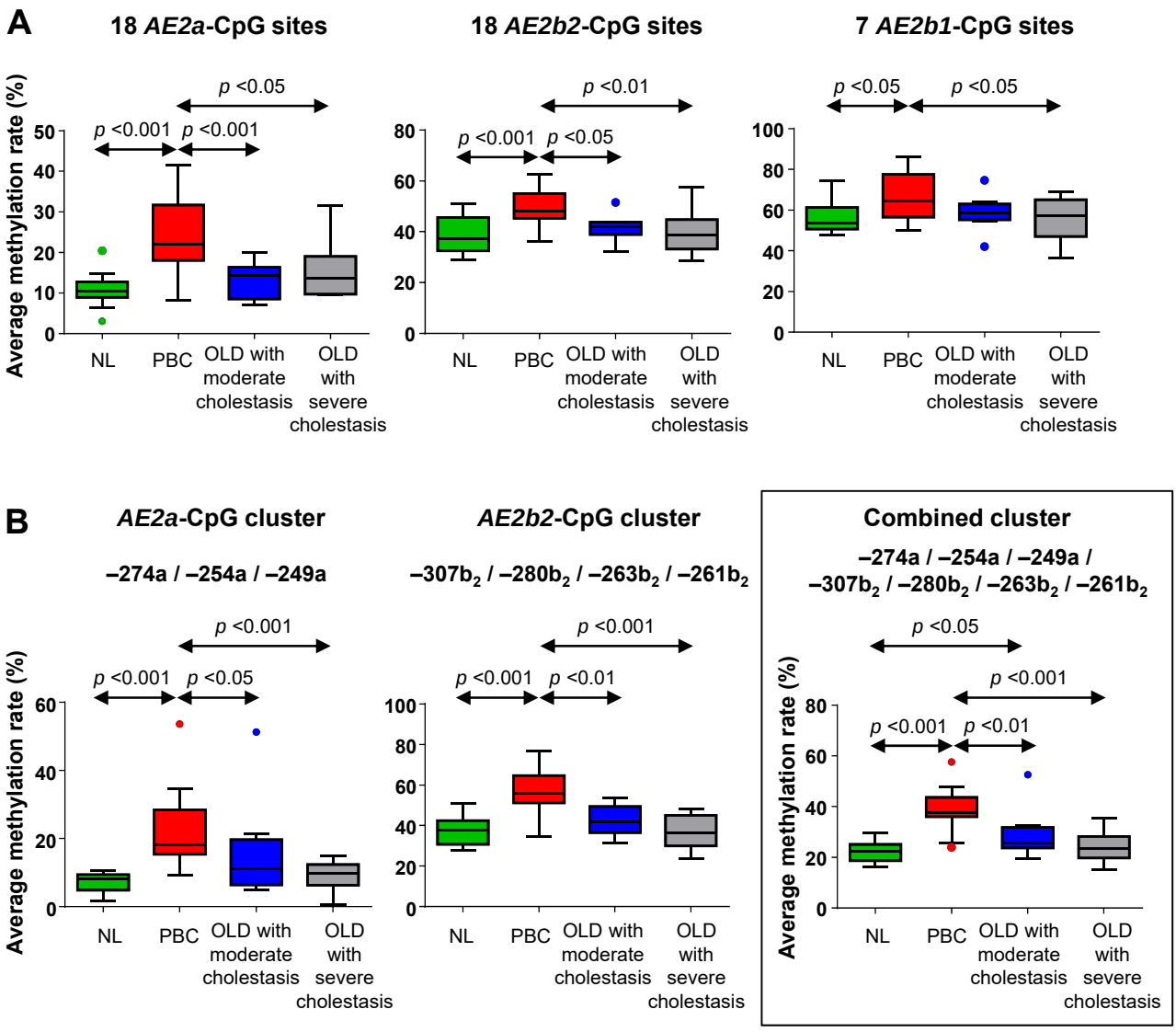

Fig. S3. *AE2* promoter regions are not hypermethylated in OLD liver samples regardless they were obtained from patients with moderate or severe cholestasis. In OLD liver specimens from severely and moderately cholestatic patients, similar average methylation rates were consistently observed for all interrogated CpG sites in *AE2* promoter regions. (A) Average CpG-methylation rates of *AE2a* and *AE2b2* proximal regions (with 18 CpG sites each) were significantly enhanced in PBC versus normal specimens and sub-grouped OLD controls. (B) Parallel data were obtained for the average CpG-methylation rates of *AE2a* and *AE2b2* minimal clusters, i.e. –274a, –254a, and –249a CpG sites, and $–307b_2$, $–280b_2$, $–263b_2$, and $–261b_2$ CpG sites, respectively (and the resultant combined cluster, right panel) when comparing PBC versus NL and sub-grouped OLD controls. 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.

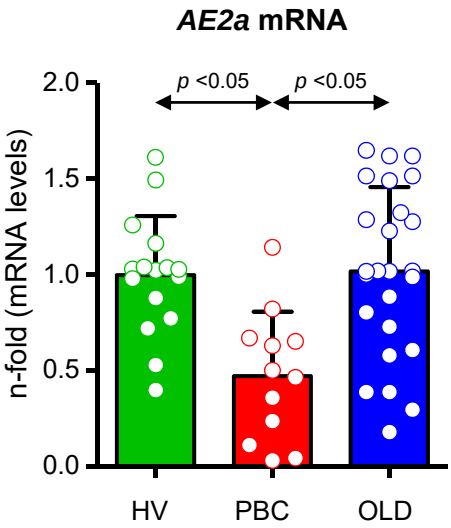

Fig. S4. Expression of *AE2a* mRNA in PBMC samples. The levels of *AE2a* mRNA were determined by real-time PCR in PBMCs from patients with PBC (n = 12), from patients with OLDs (n = 24), and in anonymized PBMC samples from HVs (n = 16). Values (normalized for GAPDH mRNA) are given as fold expression relative to mean value for HV in the respective AE2 isoform. Significant *p* values (<0.05) were obtained by Kruskall-Wallis test followed by Mann-Whitney *U*-test. HVs, healthy blood-donors/ volunteers; OLDs, other liver diseases PBC, primary biliary cholangitis; PBMCs, peripheral blood mononuclear cells.

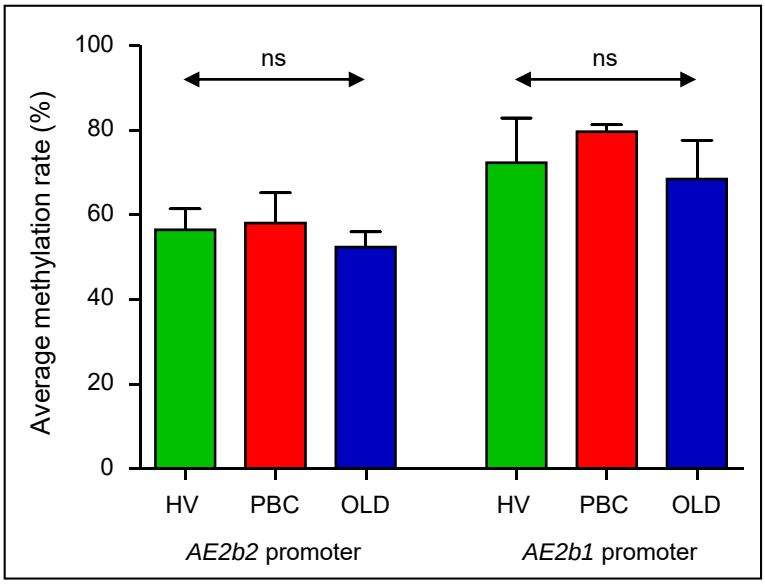

Fig. S5. Hypermethylation of *AE2* alternative promoters in PBMCs. Overall methylation rates (mean ± SD) of interrogated CpG-cytosines within proximal regions of the alternate *AE2b2* promoter (18 CpG-cytosines) and *AE2b1* (7 CpG-cytosines) promoter. Pyrosequencing was assessed on bisulphite-converted PBMC-gDNA from HVs (n = 5), from patients with PBC (n = 5) and from patients with OLDs (1 non-alcoholic fatty liver disease and 3 viral hepatitis C). ns, not significant; gDNA, genomic DNA; HVs, healthy blood-donors/ volunteers; OLDs, other liver diseases PBC, primary biliary cholangitis; PBMCs, peripheral blood mononuclear cells.

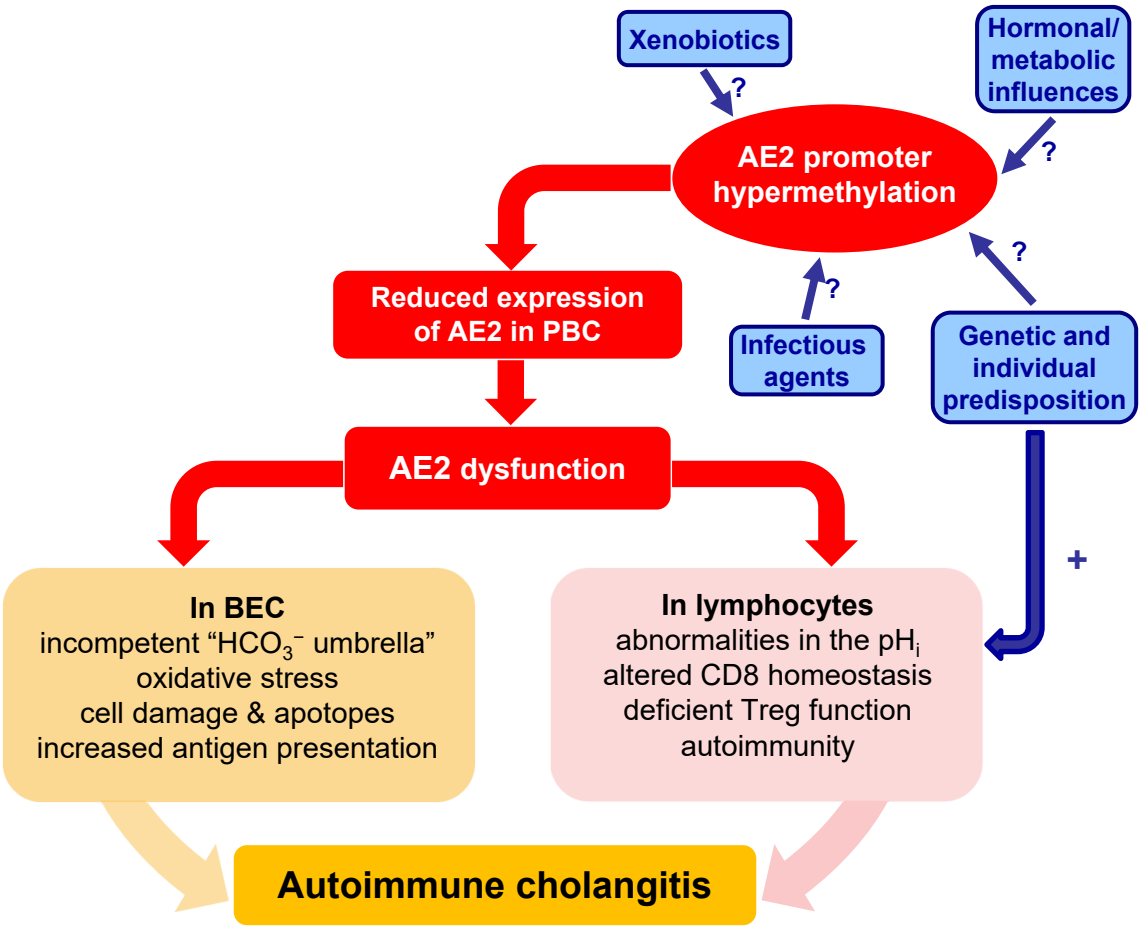

Fig. S6. Postulated role of *AE2* promoter hypermethylation and diminished *AE2* gene expression in the liver and PBMCs for PBC pathogenesis. In PBC, a series of environmental factors, in a context of genetic and individual predisposition, may lead to hypermethylation of particular *AE2* promoter regions and consequent decreased expression of AE2. According to available experimental data (cf. the main text), diminished AE2 expression in bile-duct epithelial cells (BEC) can result in incompetent ''bicarbonate umbrella'' and entry of harmful hydrophobic bile acids, oxidative stress and cell damage, which may promote apotope generation and increased antigen presentation. In lymphocytes, AE2 deficiency might result in deregulated intracellular pH (pH_i) and altered T-cell homeostasis, with enhanced immunoreactivity of cytotoxic T cells among others, and decreased Treg function. These coincidental abnormalities in both liver and PBMCs may facilitate the development of autoimmune cholangitis. Moreover, autoimmunity in patients with PBC might be fostered further by the concurrence of genetic polymorphisms associated to increased susceptibility to the disease.