Lack of hepatocellular CD10 along bile canaliculi is physiologic in early childhood and persistent in Alagille syndrome

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Many tissues, including hepatobiliary cells, express neutral endopeptidase (CD10), encoded by *MME*. Serum neutral endopeptidase activity (NEA) has been recommended as a marker of cholestasis in adults but not in children with Alagille syndrome (AGS). We investigated ontogenic and disease-related differences in the expression of CD10. CD10 was found on canalicular surfaces of hepatocytes throughout the lobule in 16 adults and in 31 children aged ≥ 24 months, with and without cholestasis, but not in 39 children aged < 24 months, with and without cholestasis. Ten AGS children aged 2 months to 6 years lacked any canalicular CD10 expression. Cholangiocyte apices and/or intrasinusoidal granulocytes marked for CD10 in all subjects. Liver membrane fractions from a child with cholestasis aged < 24 months and from 2 AGS patients aged > 24 months contained reduced levels of CD10. In contrast, AGS children and all controls expressed CD10 similarly on granulocytes. *MME* mRNA was found in the liver of children aged < 24 months and of adults, all with cholestasis, and of AGS patients. Granulocyte *MME* mRNA levels were similar among all study subjects; however, liver *MME* mRNA levels were 6- to 140-fold less than in normal adults in all cholestatic subjects, including AGS children. Methylation of the *MME* promoter was not detected in the liver of AGS children. In conclusion, hepatocytes in early childhood physiologically lack immunohistochemically detectable CD10. Reduced *MME* mRNA in AGS is not due to *MME* promoter methylation. Liver CD10 in childhood appears to undergo reduced synthesis or rapid degradation, which persists in AGS. Absence of CD10 expression thus may limit NEA as a marker of cholestasis in young patients and in AGS.

The membrane metalloendopeptidase CD10 (also known as MME, common acute lymphoblastic leukaemia antigen/CALLA, neprilysin, membrane-associated neutral endopeptidase/NEP, and enkephalinase) is a type II 95- to 100-kDa integral membrane protein identified in a wide range of normal and neoplastic tissues.[1] These include epithelia of liver, kidney, endometrium, and gastrointestinal tract, polymorphonuclear granulocytes, and lymphoid progenitor cells.[1–5] CD10 is encoded by *MME*, which is located on chromosome 3q21–q27 (Mendelian Inheritance in Man 120 520). *MME* undergoes alternative splicing in its 5′ untranslated region, generating three variant messenger RNA (mRNA) species.[3,6–8] CD10 cleaves peptides at the amino side of hydrophobic amino-acid residues, which degrades many bioactive compounds, including atrial natriuretic factor,

substance P, endothelin, interleukin-1, bradykinin, cholecystokinin, and enkephalins.[9–11] Concentrations of CD10 activity in serum can be quantified as neutral endopeptidase activity (NEA).

Cholestasis comprises a constellation of clinical, biochemical, and histological findings in which the predominant abnormality is a failure of bile flow. Cholestasis may result from secretory failure at the hepatocyte, as in various forms of progressive familial intrahepatic cholestasis.[12] Alternatively, it may result from obstruction of portions of the biliary tract, whether extrahepatic or intrahepatic, as in gallstone disease or loss of interlobular, but not septal or hilar, bile ducts that characterises Alagille syndrome (AGS).

Clinical-biochemistry findings that define cholestasis include high serum bile acid concentrations and low biliary bile

acid concentrations.[12] Cholestasis can also be associated with increased serum concentrations of γ-glutamyl transpeptidase (γ-GT) activity (γ-GTA). γ-GTA values rise when bile acids elute γ-GT from the canalicular membrane of hepatocytes; γ-GT then refluxes with bile into plasma.

CD10, like γ-GT, is located in microvilli of the canalicular membrane of hepatocytes.[1,4] Published information conflicts on whether NEA, like γ-GTA, can serve as a clinical-biochemistry marker of cholestasis. One study found that adults with cholestasis had mean NEA values 13 times higher than those in either control patients or patients with hepatocellular disease.[13] Elevated NEA was also observed in patients with stage III primary biliary cirrhosis (PBC), supporting NEA as a marker of cholestasis.[14] In contrast, children with cholestasis arising from AGS had very low NEA values, as did healthy children.[2]

During immunohistochemical study of various canalicular proteins in patients with cholestasis, we observed that in infancy and early childhood CD10 expression was not uniform at the canaliculus in patients with and without cholestasis. This prompted evaluation of the ontogeny of CD10 expression. We immunohistochemically assessed CD10 expression in livers of paediatric patients of varying age and of adults. CD10 processing was addressed in liver membrane fractions. As granulocytes express CD10, we also used fluorescence-activated cell sorting (FACS) to assess expression of CD10 by granulocytes from selected patients of various ages. To investigate any role played by body-wide ontogenic shifts in *MME* processing, mRNA was analysed in liver and granulocytes from suitable adult and paediatric patients. Finally, methylation-specific polymerase chain reaction (PCR) (MS-PCR) was used to determine if, in cholestatic liver, variation in *MME* promoter methylation was associated with variation in CD10 expression. All cohorts studied included patients with AGS.

MATERIALS AND METHODS
Clinical Information, Patients and Control Subjects
The patients and control subjects studied constituted five groups (I–V). In group I were children with non-cholestatic liver disorders ($n = 46$) from a comprehensive range of ages (1 month to 16 years, mean, 7 years; 11 < 24 months). Diagnoses included treated hepatoblastoma, α-1-antitrypsin storage disorder, hypercholesterolaemia, portal hypertension, chronic hepatitis B virus infection, and nodular regenerative hyperplasia. Group II comprised paediatric patients with cholestatic liver disease ($n = 34$; age range, 1 month to 13 years; mean, 1 year 6 months; 28 < 24 months). Diagnoses included extrahepatic biliary atresia (EHBA), progressive familial intrahepatic cholestasis, and non-familial idiopathic transient intrahepatic cholestasis ('neonatal hepatitis'). Group III was composed of paediatric patients with AGS ($n = 12$; range 2 months to 6 years 5 months; mean, 3 years 7 months; 6 < 24 months). In group IV were adults with non-cholestatic liver disorders ($n = 4$; mean age, 50

years; range, 31–69 years). All had undergone hepatic resection for metastatic large-bowel adenocarcinoma. In group V were adults ($n = 12$) with cholestatic liver disease (PBC, primary sclerosing cholangitis, benign recurrent intrahepatic cholestasis; range, 20–67 years; mean, 51 years). Liver from all patients was evaluated by light microscopy (see below). Patients from whom material was analysed by other techniques (Western blotting, FACS, reverse transcription-PCR (RT-PCR), real-time PCR, or MS-PCR) are listed in Table 1, classed as AGS, controls without cholestasis ('normal controls', NC), or controls with cholestasis (CC). Liver surplus to clinical requirements at allograft donation and granulocytes from clinically well adults provided comparison values. All samples were obtained and used with informed consent.

Microscopy and Immunohistochemical Studies
Sections routinely stained with haematoxylin/eosin and for reticulin fibres were retrieved from file, and fibrosis was scored.[15] Selected blocks were sectioned for immunostaining, using 4-μm sections on poly-L-lysine-coated slides (Polysine, Menzel, Braunschweig, Germany). Endogenous peroxidase was blocked with 1% hydrogen peroxide in methanol at room temperature for 20 min. Microwave antigen retrieval used 0.1 M citrate buffer at pH 6.0 for 3 × 5 min at 800 W, heating to boiling point with a 2-min cooling period between heating periods. Sections were then allowed to cool in buffer solution for 20 min at the end of retrieval. Tissue from each patient was then immunostained for CD10 using Novocastra NCL-L-CD10-270 monoclonal antibody (Vector Laboratories, Peterborough, UK) at a 1:150 dilution[16] and the DAKO ChemMate system (DAKO K5001, horseradish peroxidase (HRP) mouse/rabbit with diaminobenzidine chromogen; DAKO, Ely, UK). In all cases, immunostaining for two additional canalicular antigens, the type IV integral membrane protein and ATP-binding cassette transporter bile salt export pump (BSEP; polyclonal antibody raised in rabbit; 1:50 dilution; HRP rabbit/goat; a generous gift from Y Meier and B Stieger, University of Zürich),[17] and the type II integral membrane protein alanyl aminopeptidase (CD13; Novocastra NCL-CD13-304; Vector Laboratories; dilution 1:200; HRP mouse/rabbit), was conducted under the same conditions as, and in sections parallel to, those used for CD10 immunostaining. In selected cases, parallel sections were immunostained for cytokeratin 7, an antigen normally expressed by cholangiocytes but not by hepatocytes (monoclonal antibody raised in mouse; OV-TL 12/30 M 7018, DAKO; dilution 1:100; HRP mouse/rabbit). All immunostained sections were counterstained with haematoxylin using routine techniques. Two viewers assessed staining patterns independently.

Isolation of Membrane Fractions from Liver
Snap-frozen patient liver surplus to clinical diagnosis was used to prepare membrane fractions as described

Table 1 Materials selected for protein, RNA, and DNA analyses

Group, patient	Phenotype	Age	Western blotting, liver	RT-PCR, liver	FACS, granulocytes	Real-time PCR		MS-PCR, liver
						Granulocytes	Liver	
NC1	Normal adult	23 y	★	★				★
NC2	Normal adult	32 y					★	
NC3	Normal adult	35 y					★	
NC4	Normal adult	28 y					★	
NC5	Normal adult	40 y					★	
NC6	Normal child	1 y 6 mo					★	
NC7	Normal adult	51 y			★	★		
NC8	Normal adult	43 y			★	★		
NC9	Normal adult	28 y			★	★		
NC10	Normal adult	34 y			★	★		
NC11	Normal adult	31 y			★	★		
AGS1	Alagille syndrome	6 y 4 mo		★				★
AGS2	Alagille syndrome	5 y 1 mo		★				★
AGS3	Alagille syndrome	5 y 9 mo		★	★	★	★	
AGS4	Alagille syndrome	6 y 0 mo	★		★	★	★	★
AGS5	Alagille syndrome	6 y 5 mo	★				★	★
CC1	Propionic acidaemia	9 mo		★				
CC2	EHBA	7 mo		★				★
CC3	EHBA	7 mo		★				
CC4	PBC	52 y		★				★
CC5	PBC	66 y		★				★
CC6	BSEP deficiency	13 y	★		★	★	★	★
CC7	EHBA	2 mo			★	★	★	★
CC8	PBC	59 y					★	★
CC9	BSEP deficiency	10 mo	★				★	

AGS, Alagille syndrome; BSEP, bile salt export pump; CC, control with cholestasis; EHBA, extrahepatic biliary atresia; FACS, fluorescence-activated cell sorting; mo, months; MS-PCR, methylation-specific polymerase chain reaction; NC, normal control without cholestasis; PBC, primary biliary cirrhosis; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; y, years.
Analyses by Western blotting (liver), FACS (granulocytes), and PCR (liver, RT-PCR; granulocytes and liver, real-time PCR; liver, MS-PCR) are indicated by ★.

previously.[18] Tissue (approximately 1 g) in 10 volume of ice-cold 250 mM sucrose/10 mM Tris-HCl buffer (pH 7.4), supplemented with Complete Protease Inhibitor Cocktail without EDTA (Roche, Lewes, UK), was homogenised by 20 strokes in a loose glass–Teflon homogeniser. After centrifugation at 3000 g (10 min, 4°C), the post-nuclear supernatant was spun at 100 000 g (1 h, 4°C). The membrane pellet was resuspended in 100 µl of ice-cold 300 mM sucrose/10 mM HEPES buffer (pH 7.5) supplemented with Complete Protease Inhibitor Cocktail without EDTA (Roche) by 20 passages through the bore of a 23-gauge needle. Protein concentrations were measured using the DC Bradford Assay kit (Bio-Rad, Hemel Hempstead, UK) and membranes were stored at −70°C until analysis.

Western Blot Analysis

Samples of membrane fractions (100 µg each) were reduced and subjected to 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis in duplicate gels. One gel was stained with Coomassie blue to confirm uniformity of protein loading among samples. The contents of the other gel were transferred to a polyvinylidine difluoride membrane (Millipore, Watford, UK) and probed at 4°C overnight with a 1:200 dilution of NCL-CD10-270 monoclonal antibody (Vector Laboratories) in 5% skimmed-milk powder/0.1% Tween 20 in phosphate-buffered saline (PBS). After three washes in 0.1% Tween 20–PBS, the membrane was incubated at room temperature for 1 h with a 1:1000 dilution of HRP-conjugated goat anti-mouse secondary antibody (DAKO) in

5% skimmed-milk powder/0.1% Tween 20 in PBS. The membrane was washed three times in 0.1% Tween 20 in PBS and once in PBS before detection of immobilised CD10 using Enhanced Chemiluminescence-Plus (GE Healthcare, Little Chalfont, UK).

Isolation of Granulocytes from Whole Blood

Polymorphonuclear granulocytes were isolated from 10–15 ml of fresh heparinised blood using Polymorphprep (Axis Shield, Kimbolton, UK), following the manufacturer's instructions. The cells were stored at -140°C in 90% foetal calf serum/10% dimethylsulphoxide until use.

FACS Analysis

A total volume of $100 \, \mu l$ RPMI (Invitrogen Corporation, Paisley, UK) containing 1×10^5 granulocytes was added to a pre-chilled 12-ml polypropylene FACS tube, on ice. A 5-μl volume of the appropriate antibody (anti-CD10 or anti-CD13) or of the appropriate isotype control, all from BD Biosciences (Oxford, UK), was added to the cells and the samples were left on ice in the dark for 20 min. The samples were then washed twice in 2 ml of PBS and finally resuspended in $500 \, \mu l$ of fixing solution (PBS/1% paraformaldehyde). Samples were analysed using a FACS Calibur™ machine (BD Biosciences) and Cell Quest Pro™ Software (BD Biosciences), calculating percentages of cells marking for CD10 or for CD13 in a gated, isotype-controlled granulocyte population for each patient or control sample.

Isolation of Total RNA

Total RNA was isolated from 50–100 mg of each liver or from 2×10^6 granulocytes, using RNA-Bee (Biogenesis, Poole, UK), following the manufacturer's instructions. Samples were stored at the isopropanol stage of isolation. The RNA was then precipitated freshly before DNase treatment using DNase I (Promega, Southampton, UK), performed to remove any genomic DNA contamination.

RT-PCR of *MME*

MME mRNA is subject to alternative splicing, resulting in mRNA species variant in the 5′ untranslated region.[3] Primers for this experiment were therefore designed, using the mRNA sequence in GenBank accession NM_000902, to the non-variant coding region of *MME* mRNA.

Reverse transcription was used to produce first-strand cDNA using Transcriptor reverse transcriptase (Roche), at 55°C with 100 ng of the primer MME-RT-PCR (Table 2a). An initial PCR amplification was then performed using Vent DNA polymerase (New England Biolabs, Hitchin, UK), with primers MME-1F and MME-1R. The initial PCR products were then used as a template in a nested PCR amplification with the primers MME-2F and MME-2R. Abgene's DNA *Taq* polymerase was used (Epsom, UK), with 65 PCR cycles.

Levels of *β*-actin (*ACTB*) mRNA were measured as a control in each liver sample, using *ACTB*-specific PCR

Table 2a Primer sequences used for *MME* and *β-actin* (*ACTB*) mRNA RT-PCR analyses

Gene	Primer name	Sequence (5′–3′)	Product (bp)
MME	MME-RT-PCR	GCACAGTCCAGGCAATTTCAGGATT	———
	MME-1F	TTAGCAATTAAAGAAAGGATCGGC	700
	MME-1R	GCCAGAGTATGCGGTTAACTCCAT	
	MME-2F	AAACTGAATAATGAGTACCTCGAGTTG	613
	MME-2R	GAACTTTGCACAGGTGTGGTGTGGAA	
ACTB	ACTB-RT-PCR	CAGCACAATGAAGATCAAGATCAT	———
	ACTB-1F	TCTACAATGAGCTGCGTGTGGCTC	678
	ACTB-1R	GGCATTGCCGACAGGATGCAGAAGG	
	ACTB-2F	AGGAGCACCCCGTGCTGCTGAC	571
	ACTB-2R	AGACAGCACTGTGTTGGCGTACAGG	

ACTB, *β-actin*; bp, base pairs; RT-PCR, reverse transcription-polymerase chain reaction.

primers designed using the mRNA sequence in GenBank accession X00351. The reverse transcription primer used was ACTB-RT-PCR (Table 2a). An initial PCR amplification used the primers ACTB-1F and ACTB-1R. A nested PCR amplification used the primers ACTB-2F and ACTB-2R.

For both *MME* and *ACTB*, a PCR control involving RNA that had not undergone reverse transcription reaction was performed with each sample, to check for genomic DNA contamination. A PCR negative control was also performed. PCR products were analysed on 1.5% agarose gels.

Sequencing of PCR Products

PCR product ($30 \, \mu l$) was purified using the High Pure Purification Kit (Roche), following the manufacturer's instructions. The eluted products were sequenced using Big Dye version 3.1 (Applied Biosystems, Warrington, UK) and data were collected on an ABI 3100-Avant Sequencer (Applied Biosystems). Chromatograms were analysed using Sequencher software (Gene Codes, Ann Arbor, MI, USA).

Real-Time PCR of *MME*, *ANPEP*, and *ABCB11*

DNase-treated total RNA (100 ng) from all patients and controls was used as a template in a reverse transcription reaction using Transcriptor reverse transcriptase (Roche) at 50°C with 100 ng of random hexamer primers (Invitrogen Corporation).

Each first-strand cDNA ($1 \, \mu l$) from liver and granulocytes was assessed in triplicate for levels of *MME* using ABI Taq-Man® Gene Expression Assay for *MME* (Applied Biosystems; Assay ID: Hs00153519_m1). Levels of the gene encoding BSEP, *ABCB11*, were measured as a control in liver using ABI TaqMan Gene Expression Assay for *ABCB11* (Applied Biosystems; Assay ID: Hs00184824_m1). Levels of the gene encoding CD13, *ANPEP*, were measured as a control in

Table 2b Primer sequences used for *MME* MS-PCR analysis

Promoter status	Primer name	Sequence (5′–3′)	Product (bp)
Unmethylated	MME-U-F	TGGGTGGATGTATGGATTGAGAGGTG	200
	MME-U-R	TCCCACAAACTCCCAACACCCTAACA	
Methylated	MME-M-F	GCGGATGTACGGATTGAGAGGCG	195
	MME-M-R	CCACAAACTCCCAACGCCCTAACG	

bp, base pairs; MS-PCR, methylation-specific polymerase chain reaction.

granulocytes using ABI TaqMan Gene Expression Assay for *ANPEP* (Applied Biosystems; Assay ID: Hs00174265_m1). 18S ribosomal RNA, an endogenous control, was measured using ABI TaqMan Endogenous Control Assay (Applied Biosystems). Genomic DNA contamination was excluded for all genes in each sample via a PCR control involving RNA that had not undergone reverse transcription reaction. PCR negative controls were also performed.

Data were collected on an ABI Prism 7000 SDS machine (Applied Biosystems) and analysed with ABI SDS software, RQ study application version 1.2.3 (Applied Biosystems), using the comparative cycle threshold method. Data were normalised to 18S rRNA expression (delta [d] cycle threshold) and plotted as fold differences in mean cycle threshold values (dd cycle threshold) between patients and controls (liver calibrator, subject NC4; granulocyte calibrator, subject NC9).

Methylation Status of the *MME* Promoter in Liver

Genomic DNA was isolated from 150–300 mg of liver using the QIAGEN DNA Mini kit, following the manufacturer's instructions. A 2-μg weight of genomic DNA was then subjected to bisulphite treatment.[19] The treated DNA was subjected to MS-PCR using the primers MME-U-F and MME-U-R for an unmethylated allele and MME-M-F and MME-M-R for a methylated allele of the exon 2 *MME* promoter (Table 2b).[20] The primers encompassed this major transcription start site of *MME*. PCR was performed using GoTaq DNA polymerase (Promega) with 35 cycles. Products were analysed on a 2.5% agarose gel.

RESULTS

Immunohistochemical Evaluation of CD10 Expression in the Liver

In adults with and without cholestasis (groups IV and V, respectively), fine canalicular CD10 marking was present throughout the hepatic lobule (Figure 1a). It was accentuated immediately adjacent to portal tracts and lessened towards centrilobular areas, possibly reflecting organisational heterogeneity of hepatocyte function within the lobule. In materials from 39 children aged <24 months, both with and without cholestasis (groups I and II, respectively), such

definite marking was observed in just a single case (EHBA; age 23 months), with only minimal patchy staining at best in the remaining cases (Figure 1b). In materials from children aged >24 months (groups I and II), the adult pattern of CD10 staining was observed (Figure 1c) in all but two subjects, again with periportal accentuation in a number of cases. The two exceptions had idiopathic chronic hepatitis (age 26 months) and EHBA (age 25 months). In children aged >36 months or in adults, CD10 expression was not appreciably stronger than in children aged 24–36 months.

From at least age 2 months, prominent linear staining of the luminal aspect of bile duct epithelium was evident (marking at cholangiocyte apices). Where bile ducts were present, this acted as a convenient internal control, together with CD10 marking of 'passenger' leucocytes/granulocytes within the specimen (Figure 1d).

The luminal aspect of biliary epithelium stained for CD10 in both adult and paediatric cases (Figure 1a and b). Some marking along luminal margins was also observed in regions of ductular proliferation, where hepatocytes appeared in transition towards a more biliary phenotype (ectopic expression of cytokeratin 7 by 'transitioning' hepatocytes was also observed in a similar distribution). Liver of five patients marked perisinusoidally for CD10.

AGS patients of all ages, including >24 months (group III), had no detectable canalicular CD10 expression (Figure 1d). To correlate CD10 expression with the degree of fibrosis,[21] sections stained for reticulin fibres were assessed. Fibrosis scores for selected patients are shown in Table 3, along with CD10 and control-antigen (CD13 and BSEP) staining results. CD10 staining was deficient in the canalicular membrane of children aged <24 months and in that of AGS patients at all ages studied. Fibrosis in AGS patient livers was mild or moderate at worst; adults with PBC had frank cirrhosis. Cirrhosis did not, in PBC, adversely affect CD10 expression.

CD10 Expression in Liver Membrane Fractions

Membrane fractions were prepared from livers of various subjects (Table 1) to visualise CD10 protein by Western blot analysis. All samples had detectable CD10 protein of 100 kDa in size (Figure 2), as expected for the mature processed protein.[1] However, compared with normal adult liver, the liver from a child aged 10 months (CC9) had less detectable CD10 protein. Furthermore, compared with normal liver (NC1, aged 23 years) and liver from a child with BSEP deficiency (CC6, 13 years), AGS patients (AGS5, 6 years 5 months; AGS4, 6 years 0 months) had considerably reduced levels of CD10 protein.

CD10 and CD13 Expression on Granulocytes

To enumerate cells expressing CD10 among granulocytes isolated from patients and controls, FACS analysis was performed (Table 1; Figure 3). In all patients but one, proportions of cells expressing CD10 were comparable to those of
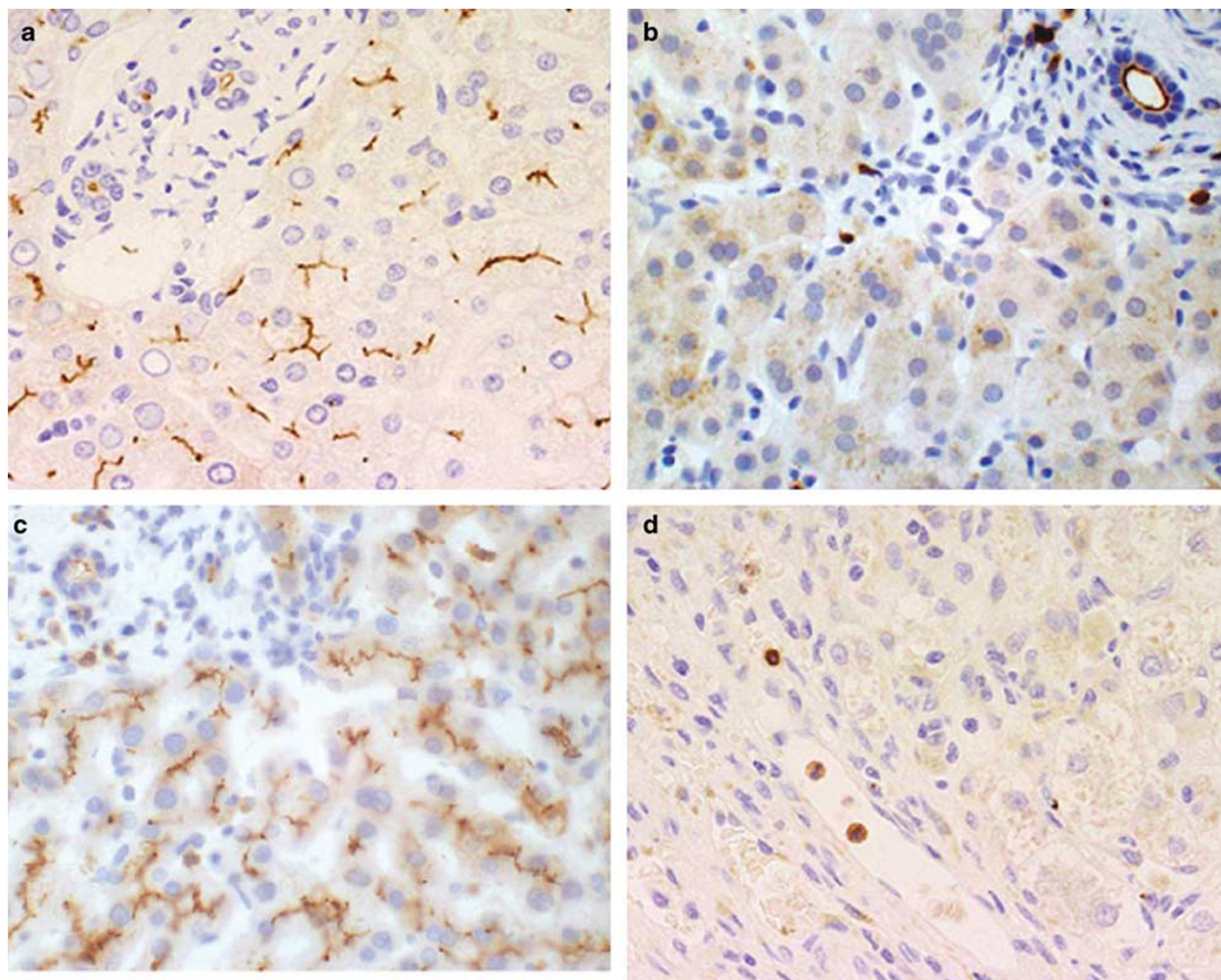
Figure 1 CD10 expression in adult and paediatric liver specimens. All images: anti-CD10/haematoxylin. (**a**) CD10 marking in normal adult liver, aged 38 years. CD10 marks canaliculi (linear network) and cholangiocyte apices (centres of rosettes). Original magnification × 400. (**b**) Canalicular marking for CD10 is absent in the liver of patients aged <24 months. Liver shown is from a representative patient, aged 2 months, with BSEP deficiency. Cholangiocyte apices and scattered leucocytes mark well. Original magnification × 400. (**c**) CD10 marks canaliculi in liver of patients aged >24 months. Liver shown is from a representative patient, aged 32 months, with α-1-antitrypsin storage disorder. Cholangiocyte apices and a few leucocytes also mark well. Original magnification × 400. (**d**) Passenger granulocytes in Alagille-syndrome liver that lacks both bile ducts and canalicular CD10 expression serve as internal controls. Liver shown is from a patient aged 6 years. Original magnification × 400.

normal controls. The exception was CC7 (EHBA, aged 2 months), in whom CD10 expression was 8% reduced. Findings in this very young patient may reflect ontogenic regulation of CD10 in this cell population. Values for granulocyte CD13 expression were highly consistent among patients and controls except in AGS4 (expression 4% reduced).

MME mRNA Detection in Liver

RT-PCR was used to determine if *MME* mRNA was transcribed and processed in livers of patients with deficient CD10 protein levels (Tables 1 and 2a; Figure 4a and b). As shown in Figure 4a, *MME* mRNA was amplified as a PCR product in liver from five cholestatic patients (CC1–CC5) and in a 'normal control' liver without cholestasis (NC1). A *MME* PCR product also was detected in three AGS patients

(AGS1–AGS3; Figure 4b). *ACTB* control mRNA was amplified in all liver samples. Resultant *MME* PCR products were sequenced; results confirmed that the products originated from *MME* and that mRNA splicing was normal at the region amplified (data not shown).

Quantitative Values for *MME* mRNA in Liver and Granulocytes

Real-time PCR was used to quantitate levels of *MME* mRNA in liver and granulocyte samples from patients and controls (Table 1; Figures 5 and 6). Figure 5 shows that while *ABCB11* mRNA levels in the liver remained similar among all subjects, levels of *MME* were decreased 6- to 140-fold in cholestatic-patient livers (AGS2–AGS5 and CC4–CC9) compared with 'normal control' adult livers without cholestasis (NC2–NC6).

Table 3 Fibrosis scoring and findings on immunohistochemical staining for antigens CD10, CD13, and BSEP in selected patients

Group (age)	Fibrosis score	Immunohistochemistry results		
		CD10	CD13	BSEP
AGS1 (6 y 4 mo)	2	Marks a few endothelial cells, leucocytes, and adluminal margins of residual cholangiocytes	Well preserved at canalicular margins ('canaliculi mark')	Canaliculi mark
AGS2 (5 y 1 mo)	3	Limited to scant sinusoidal endothelium	Canaliculi mark	Canaliculi mark
AGS3 (5 y 9 mo)	3	Leucocyte staining only	Canaliculi mark	Canaliculi mark
AGS4 (6 y 0 mo)	2	Leucocyte staining only	Canaliculi mark	Canaliculi mark
AGS 5 (6 y 5 mo)	3	Leucocyte staining only	Canaliculi mark	Canaliculi mark
CC1 (9 mo)	1	Limited to cholangiocyte apices	Well preserved at canalicular margins and cholangiocyte apices ('canaliculi and cholangiocytes mark')	Canaliculi mark
CC2 (7 mo)	4	Limited to cholangiocyte apices	Canaliculi and cholangiocytes mark	Canaliculi mark
CC3 (7 mo)	4	Limited to cholangiocyte apices	Canaliculi and cholangiocytes mark	Canaliculi mark
CC4 (52 y)	4	Canaliculi and cholangiocytes mark	Canaliculi and cholangiocytes mark	Canaliculi mark
CC5 (66 y)	4	Canaliculi and cholangiocytes mark	Canaliculi and cholangiocytes mark	Canaliculi mark
CC6 (13 y)	1	Canaliculi mark, but patches of less dense staining in nodules	Canaliculi and cholangiocytes mark	No staining (mutation in *ABCB11* demonstrated on both alleles)[a]
CC7 (2 mo)	3	Limited to cholangiocyte apices	Canaliculi and cholangiocytes mark	Canaliculi mark
CC8 (59 y)	4	Canaliculi and cholangiocytes mark, but patches of less dense staining in nodules	Canaliculi and cholangiocytes mark	Canaliculi mark, but patches of less dense staining in nodules
CC9 (10 mo)	1	Limited to cholangiocyte apices	Canaliculi and cholangiocytes mark	No staining (mutation in *ABCB11* demonstrated on both alleles)[a]

AGS, Alagille syndrome; BSEP, bile salt export pump; CC, control with cholestasis; mo, months; y, years.
[a]Predicted effects on BSEP: DC6, R1268Q homozygote; DC9, N490D/V284A heterozygote.
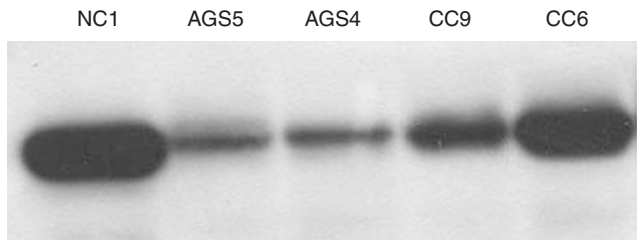

Figure 2 CD10 in liver membrane fractions. Western blot of liver without cholestasis (NC1, aged 23 years), older AGS patients aged >24 months (AGS5, 6 years 5 months; AGS4, 6 years 0 months;), and livers with cholestasis; an infant with BSEP deficiency and cholestasis (CC9, aged 10 months), and an adolescent with BSEP deficiency and cholestasis (CC6, aged 13 years). Less CD10 protein is present in AGS membrane fractions.
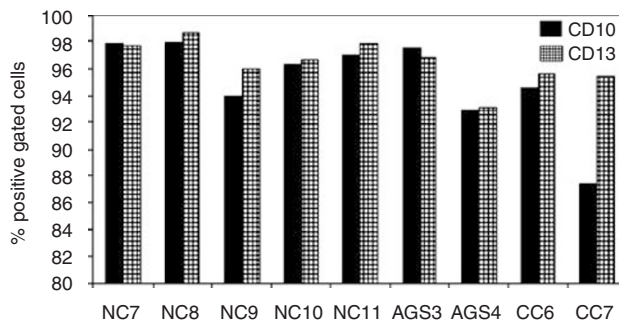

Figure 3 Percentages of granulocytes expressing CD10 and CD13 by FACS analysis. CD10 (black bars) and CD13 (hatched bars) in NC7–NC11, healthy adults without cholestasis; AGS3 and AGS4, AGS patients aged >24 months; CC6, aged 13 years, and CC7, aged 2 months, control patients with cholestasis.

The 22% decrease in *MME* found in NC6 (aged 1 year 6 months) is ascribed to ontogenic regulation of *MME* transcription in a normal liver.

Figure 6 shows that *MME* mRNA levels in granulocytes were relatively consistent among all subjects, despite varying ages. Levels of mRNA from the gene encoding CD13, *ANPEP*,
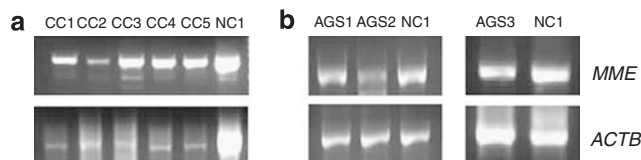
Figure 4 *MME* mRNA in liver. (**a**) Control patients with cholestasis (CC1–CC5) and patient without cholestasis (NC1) and (**b**) AGS patients (AGS1–AGS3) and patient without cholestasis (NC1). β-Actin (*ACTB*) mRNA was amplified as a control. Both *MME* and *ACTB* mRNA could be amplified in all samples.
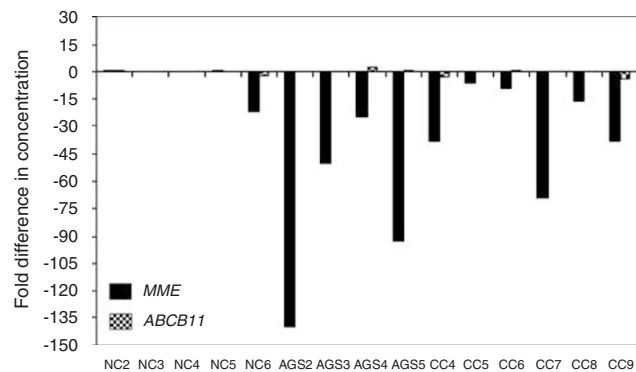

Figure 5 Quantitation of *MME* and *ABCB11* mRNA levels in the liver. *MME* (black bars) and *ABCB11* (chequered bars) levels were quantitated by real-time PCR in the liver of AGS patients (AGS2–AGS5) and various controls (cf. Tables 1 and 3, patients without cholestasis, NC2–NC6; and patients with cholestasis, CC4–CC9). *MME* mRNA levels are strikingly low in AGS patients and in CC4 (PBC, aged 52 years), CC7 (EHBA, aged 2 months), and CC9 (BSEP deficiency, aged 10 months). *ABCB11* mRNA levels are not substantially decreased *vs* non-cholestatic controls.
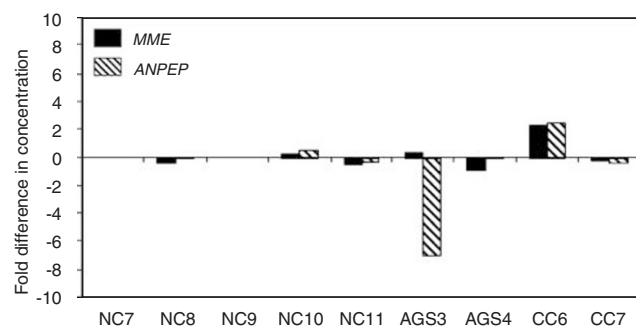

Figure 6 Quantitation of *MME* and *ANPEP* mRNA levels in granulocytes. *MME* (black bars) and *ANPEP* (diagonally striped bars) mRNA levels were quantitated by real-time PCR, in granulocytes from AGS patients (AGS3 and AGS4) and various controls (cf. Tables 1 and 3, adults without cholestasis, NC7–NC11; adolescent with cholestasis and BSEP deficiency, CC7, aged 13 years; and infant with cholestasis and EHBA, CC9, aged 2 months). *MME* mRNA levels do not vary substantially among subjects, although *ANPEP* mRNA levels are somewhat decreased in one AGS patient and slightly increased in the adolescent with cholestasis.

were measured as a control. This gene was expressed at a level similar to that for *MME* in all samples, except one AGS patient (AGS3, 5 years 9 months) in whose liver a seven-fold
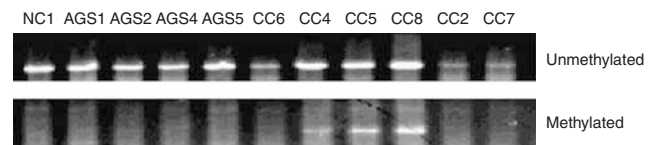

Figure 7 MS-PCR analysis of *MME* promoter in the liver. Primers specific for an unmethylated allele *vs* a methylated allele of the *MME* promoter were used for MS-PCR analysis. Methylation is present only in PBC patients (CC4, CC5, and CC8), and is not found in AGS patients.

decrease in *ANPEP* mRNA was observed. The data overall demonstrate a decrease in *MME* mRNA levels in liver from young and cholestatic patients, a trend not found in the same patients' granulocytes.

Methylation Status of the *MME* Promoter in Liver

Bisulphite treatment of genomic DNA from patient and control liver followed by MS-PCR (Tables 1 and 2b) was performed to determine if reduced *MME* mRNA levels resulted from *MME* promoter methylation at a CpG island located at the major transcription start site of the gene.[20] All patients analysed had predominantly unmethylated alleles of the *MME* promoter. The only individuals who also had a methylated form of the promoter were those with PBC (CC4, CC5, and CC8; Figure 7).

DISCUSSION

Although the fluid within the biliary canaliculus and that at the ampulla of Vater are both called bile, they are very different substances. To understand both post-secretory modification of bile and paracrine signalling within the hepatobiliary tract,[22] the proteins expressed at the canalicular membrane and the cholangiocyte apex must be identified and functionally analysed, and how their expression is regulated must be described.

CD10 is a type II integral membrane protein, encoded by *MME*, that cleaves extracellular bioactive peptides at various body sites.[1,9–11] Its function along canalicular and bile-duct membranes is not defined, but may be presumed to involve post-secretory modification of bile. In this study we demonstrated that in infancy and early childhood, CD10 expression is physiologically absent from the canalicular membrane of hepatocytes, but is present on cholangiocyte apices and on granulocyte surfaces. Furthermore, even beyond 24 months of age, CD10 expression was deficient specifically along the canaliculi of AGS patients. This was accompanied by dramatically reduced levels of CD10 protein in membrane fractions isolated from livers of such patients. *MME* mRNA was detected in the livers of infants and adults with numerous cholestatic liver diseases, including AGS, albeit at levels lower than in non-cholestatic control adult livers, but was found at similar/comparable levels in granulocytes from these subjects. The liver of AGS patients, as well as those with BSEP deficiency and EHBA, had no detectable methylation of the *MME* promoter. We therefore conclude

that CD10 undergoes ontogenic regulation, with inhibited expression, in hepatocytes for the first 2 years of life and that canalicular expression of CD10 is lacking in AGS both in early childhood and thereafter. This differs from CD10 expression and its ontogenic regulation in granulocytes, where near-adult levels of CD10 protein and *MME* mRNA are reached by 2 months of age in both comparison subjects and AGS patients.

CD10 expression reportedly falls as fibrosis increases.[21] However, while CD10 was absent from canaliculi in some foci of hepatocellular nodular regeneration, no association between increasing fibrosis scores and loss of CD10 expression was observed; AGS patients lacking CD10 expression had grade 2 or grade 3 fibrosis (scale 0–4), but canaliculi from adults without AGS and with similar or worse fibrosis expressed CD10 well.[15] These observations suggest that fibrosis alone does not account for lack of canalicular CD10 expression in AGS. One of our control antigens, BSEP, the main bile salt transporter of human liver, similarly situated in the canalicular membrane,[23,24] also marked patchily in regenerative nodules. CD13 expression appeared more robust, serving to identify canaliculi as well as cholangiocyte apices throughout these materials.[25]

None of the patients whom we studied was known to have hepatitis C virus infection, while most of those in whom fibrosis was associated with loss of canalicular CD10 expression were thus infected.[21] These disparate findings suggest that loss of canalicular CD10 expression in patients with fibrosis owing to hepatitis C virus infection may not be simply due to increasing fibrosis. Rather, viral infection may affect expression of CD10. Such loss of CD10 expression may not be due to chronic viral infection alone, as CD10 loss reportedly was much less pronounced in patients with hepatitis B virus infection.[21]

Our work sheds light on the ontogenic regulation of both CD10 and *MME* in granulocytes. Newborn babies have 55% lower levels of CD10 expression on granulocytes than do adults.[26] In our study, CD10 cell surface expression on these cells at age 2 months was only 8% less than that on granulocytes of adults. *MME* mRNA levels, measured by real-time PCR, in granulocytes were similar at age 2 months and in adults. Adult levels of *MME* transcription in granulocytes must therefore be attained between birth and age 2 months, with protein levels following closely behind. Values for CD13 expression on granulocytes of newborn children are reportedly half those on granulocytes of adults.[26] Our results show that at age 2 months, adult levels of both *ANPEP* mRNA and CD13 protein have been reached.

In the liver, however, we have shown that CD10 canalicular expression is not observed until at least age 24 months, and that this correlates with a decrease in *MME* mRNA levels until that time. By contrast, cholangiocyte apices in children aged <24 months mark for CD10. This suggests that any ontogenic decrease in hepatobiliary CD10 expression is hepatocyte specific. The membrane fractions isolated from livers of patients and controls combine canalicular and cholangiocyte membranes. It is possible that mature CD10, detected by Western blot analysis, and *MME* mRNA actually originate only from cholangiocytes.

In patients aged >24 months, except those with AGS, liver *MME* mRNA generally was downregulated in the setting of cholestasis, while CD10 protein levels appeared unchanged (as assessed immunohistochemically and by Western blotting). CD10 protein levels may be in excess in the liver after age 24 months and thus be unaffected by mRNA downregulation during cholestasis. Subapical vesicles in hepatocytes could allow intracellular maintenance of large pools of mature CD10, as documented for BSEP.[27] In contrast, liver CD10 protein levels were dramatically reduced in AGS patients aged >24 months, compared with age-matched cholestatic controls, suggesting instability of protein product or impaired translation of mRNA into protein.

Functional correlations of postnatal shifts in liver organisation remain largely unexplained. Remodelling from two-cell to one-cell hepatocyte plates, for example, occurs as canalicular CD10 expression begins. The mechanisms of regulation of CD10 remain to be assessed; in cholestasis, gene transcription could be reduced via methylation of the promoter region of *MME*, as seen in prostate cancer[20] and in hepatocellular carcinoma of the rat.[28] However, the major *MME* promoter at exon 2 was methylated neither in AGS patients nor in BSEP-deficient and EHBA patients. Methylation of this promoter therefore did not account for the reduced *MME* mRNA levels observed. Reduced *MME* mRNA levels in cholestasis and in early youth could be due to downregulation of a positive-acting transcription factor or to upregulation of a negative-acting one. The transcription factor CBF/NF-Y has been shown to bind selectively in a tissue-specific manner to the *MME* promoter region analysed for methylation in this study.[20,29] Whether the roles of such factors in the transcription of *MME* differ between hepatocytes and granulocytes certainly warrants future investigation. *MME* promoter methylation in PBC also awaits study.

Any model of the effects of *JAG1/NOTCH2* mutations that underlie AGS[30,31] must account for disruption of ontogenic regulation of CD10 expression, with decreases in CD10 protein. Further study is required to determine if in AGS degradation of CD10 via the ubiquitin–proteasome pathway (as with sodium taurocholate co-transporting polypeptide[32]) is increased, or *MME* mRNA is directed away from ribosomes, thus preventing its translation and reducing *de novo* synthesis of CD10.[33] A lack of demonstrable CD10 protein in an adult woman was reported by Debiec et al,[34] who described renal disease in her offspring. This was traced to transplacental transmission of maternal antibodies against CD10, which was expressed on the infant glomerulus. The infant had no hepatobiliary disease (PM Ronco, personal communication); indeed, physiologic failure to express CD10 along the immature canaliculus would preclude immune-mediated injury to hepatocytes. Of interest is that the mother

also had no recognised hepatobiliary disease. Canalicular CD10 expression is *prima facie* not required for normal hepatobiliary function in infants and young toddlers, nor was it necessary for clinical health in the mother described. That absence of CD10 substantially contributes to bile duct loss in AGS is improbable, as such loss develops well before the second birthday.[30]

Yet CD10 may play an important role in the pathophysiology of cholestasis, if it modulates the levels or activities of bioactive substances in both bile and the circulating plasma. Endogenous opioids, catabolised by CD10,[10] are implicated in the proliferative ductular reaction of obstructive cholestasis.[35] Lack of CD10 in older AGS patients may reflect or contribute to the evolution of the disorder. Detailed analysis of bile from such patients, comparing its constituents with those of normal bile and of bile from cholestatic patients with canalicular expression of CD10, might prove rewarding, as might comparison of bile composition between infants not expressing CD10 and older children expressing CD10.

Clinical-biochemistry tests in patients with liver disease are of great value in directing the use and interpreting the results of other diagnostic tests, notably imaging and liver biopsy. Attention is required, however, to the mechanisms that underlie rises or falls in values for individual analytes, and immunohistochemical and molecular analyses may increase the precision with which such tests can be employed. The serum markers for cholestasis currently most used are γ-GTA and alkaline phosphatase activity. Alkaline phosphatase is normally located in the canalicular membrane of hepatocytes and in apical cholangiocyte cytoplasm; in patients with PBC, however, alkaline phosphatase was also found in the basolateral membrane of hepatocytes and throughout the cytoplasm of cholangiocytes, in association with increased alkaline-phosphatase protein and mRNA levels in liver tissue.[36] In some forms of intrahepatic cholestasis, γ-GT expression along canaliculi appears disordered or lacking, and γ-GTA in patients with those disorders does not rise despite cholestasis.[37,38]

Our study demonstrates that variation in hepatobiliary CD10 expression may account for variation in conclusions drawn from previous studies of whether NEA can mark cholestasis. Swain et al[13] and Horvát-Karajz et al[14] concluded that NEA could mark cholestasis, whilst Janas et al[2] found no difference in NEA between cholestatic patients and controls. The cholestatic patients studied by Janas et al[2] had AGS. As AGS patients lack CD10 in the canalicular membrane, we infer that CD10 could not be eluted into these patients' bile and that their values for NEA could not rise. Our results also predict that values for NEA will not rise in children aged <24 months with cholestasis, as their hepatocytes, like those of patients with AGS, do not express CD10.

In conclusion, the hepatocytes of children in early childhood physiologically fail to express CD10. In AGS, no CD10 is detected even beyond infancy; whilst *MME* mRNA levels are reduced, they are not more so than in cholestatic controls

that express CD10. Methylation of the *MME* promoter does not explain reduced *MME* mRNA levels in AGS or cholestasis, and other defects in transcriptional regulation are likely. CD10 is thus inferred to undergo reduced synthesis or rapid degradation in the liver of AGS patients and of young children. Lack of CD10 expression likely precludes an increase in NEA despite cholestasis. NEA as a marker of cholestasis is therefore predicted to be of limited value in young patients and in AGS.

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