



## UvA-DARE (Digital Academic Repository)

### Mutations in SRD5B1 (AKR1D1), the gene encoding Delta(4)-3-oxosteroid 5 beta-reductase, in hepatitis and liver failure in infancy

Lemonde, H.; Custard, E.J.; Bouquet, J.; Duran, M.; Overmars, H.; Scambler, P.J.; Clayton, P.T.

**DOI**

[10.1136/gut.52.10.1494](https://doi.org/10.1136/gut.52.10.1494)

**Publication date**

2003

**Published in**

Gut

[Link to publication](#)

**Citation for published version (APA):**

Lemonde, H., Custard, E. J., Bouquet, J., Duran, M., Overmars, H., Scambler, P. J., & Clayton, P. T. (2003). Mutations in SRD5B1 (AKR1D1), the gene encoding Delta(4)-3-oxosteroid 5 beta-reductase, in hepatitis and liver failure in infancy. *Gut*, *52*, 1494-1499. <https://doi.org/10.1136/gut.52.10.1494>

**General rights**

It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

**Disclaimer/Complaints regulations**

If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: <https://uba.uva.nl/en/contact>, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.

*UvA-DARE is a service provided by the library of the University of Amsterdam (<https://dare.uva.nl>)*

# Mutations in *SRD5B1* (*AKR1D1*), the gene encoding $\Delta^4$ -3-oxosteroid 5 $\beta$ -reductase, in hepatitis and liver failure in infancy

H A Lemonde, E J Custard, J Bouquet, M Duran, H Overmars, P J Scambler, P T Clayton

*Gut* 2003;52:1494–1499

**Background:** A substantial group of patients with cholestatic liver disease in infancy excrete, as the major urinary bile acids, the glycine and taurine conjugates of 7 $\alpha$ -hydroxy-3-oxo-4-cholenoic acid and 7 $\alpha$ ,12 $\alpha$ -dihydroxy-3-oxo-4-cholenoic acid. It has been proposed that some (but not all) of these have mutations in the gene encoding  $\Delta^4$ -3-oxosteroid 5 $\beta$ -reductase (*SRD5B1*; *AKR1D1*, OMIM 604741).

**Aims:** Our aim was to identify mutations in the *SRD5B1* gene in patients in whom chenodeoxycholic acid and cholic acid were absent or present at low concentrations in plasma and urine, as these seemed strong candidates for genetic 5 $\beta$ -reductase deficiency.

**Patients and subjects:** We studied three patients with neonatal onset cholestatic liver disease and normal  $\gamma$ -glutamyl transpeptidase in whom 3-oxo- $\Delta^4$  bile acids were the major bile acids in urine and plasma and saturated bile acids were at low concentration or undetectable. Any base changes detected in *SRD5B1* were sought in the parents and siblings and in 50 ethnically matched control subjects.

**Methods:** DNA was extracted from blood and the nine exons of *SRD5B1* were amplified and sequenced. Restriction enzymes were used to screen the DNA of parents, siblings, and controls.

**Results:** Mutations in the *SRD5B1* gene were identified in all three children. Patient MS was homozygous for a missense mutation (662 C>T) causing a Pro198Leu amino acid substitution; patient BH was homozygous for a single base deletion (511 delT) causing a frame shift and a premature stop codon in exon 5; and patient RM was homozygous for a missense mutation (385 C>T) causing a Leu106Phe amino acid substitution. All had liver biopsies showing a giant cell hepatitis; in two, prominent extramedullary haemopoiesis was noted. MS was cured by treatment with chenodeoxycholic acid and cholic acid; BH showed initial improvement but then deteriorated and required liver transplantation; RM had advanced liver disease when treatment was started and also progressed to liver failure.

**Conclusions:** Analysis of blood samples for *SRD5B1* mutations can be used to diagnose genetic 5 $\beta$ -reductase deficiency and distinguish these patients from those who have another cause of 3-oxo- $\Delta^4$  bile aciduria, for example, severe liver damage. Patients with genetic 5 $\beta$ -reductase deficiency may respond well to treatment with chenodeoxycholic acid and cholic acid if liver disease is not too advanced.

See end of article for authors' affiliations

Correspondence to: Professor P T Clayton, Biochemistry, Endocrinology, and Metabolism Unit, Institute of Child Health, 30 Guilford St, London WC1N 1 EH, UK; p.clayton@ich.ucl.ac.uk

Accepted for publication 18 June 2003

In 1988, Clayton *et al* and Setchell *et al* reported that, in some infants with liver disease, the major urinary bile acids were the glycine and taurine conjugates of 7 $\alpha$ -hydroxy-3-oxo-4-cholenoic acid and 7 $\alpha$ ,12 $\alpha$ -dihydroxy-3-oxo-4-cholenoic acid.<sup>1,2</sup> This suggested reduced activity of the enzyme responsible for saturating the  $\Delta^4$  double bond during the synthesis of bile acids from cholesterol (that is,  $\Delta^4$ -3-oxosteroid 5 $\beta$ -reductase deficiency). However, it was not clear which, if any, of these patients had defects in the gene encoding the 5 $\beta$ -reductase enzyme. Some clearly had a recognised cause of liver disease such as tyrosinaemia or hepatitis B,<sup>1</sup> suggesting that their "5 $\beta$ -reductase deficiency" was secondary to liver damage rather than a primary genetic defect. However, Shneider *et al* described excretion of 3-oxo- $\Delta^4$  bile acids as the major urinary bile acids in two unrelated infants with features of neonatal haemochromatosis and they suggested that 5 $\beta$ -reductase deficiency was the cause of neonatal haemochromatosis.<sup>3</sup>

In 1994, Kondo *et al* reported the cDNA sequence for human 5 $\beta$ -reductase.<sup>4</sup> Sumazaki *et al* used this information to investigate the possibility of primary 5 $\beta$ -reductase deficiency in a Japanese neonate with liver failure and 3-oxo- $\Delta^4$  bile aciduria.<sup>5</sup> Using liver tissue, they sequenced the entire cDNA for the enzyme and showed that it was normal, effectively excluding genetic 5 $\beta$ -reductase deficiency. Meanwhile, Clayton *et al* described a cholestatic infant in whom the

presence of large amounts of 3-oxo- $\Delta^4$  bile acids in plasma and urine was associated with low concentrations of chenodeoxycholic acid and cholic acid.<sup>6</sup> This infant had failed to improve on ursodeoxycholic acid therapy but liver function returned to normal on replacement of the natural primary bile acids, chenodeoxycholic acid and cholic acid. It was argued that this girl probably did have a primary genetic 5 $\beta$ -reductase deficiency. This paper describes the identification of a homozygous missense mutation in her 5 $\beta$ -reductase gene and identification of different homozygous mutations in the 5 $\beta$ -reductase genes of two other infants, both of whom had liver disease severe enough to require transplantation.

## PATIENTS AND METHODS

All studies were undertaken with permission from the Research Ethics Committee of Great Ormond Street Hospital/Institute of Child Health.

**Abbreviations:** AST, aspartate aminotransferase; ALT, alanine aminotransferase;  $\gamma$ -GT,  $\gamma$ -glutamyl transpeptidase; ALP, alkaline phosphatase; APTT, activated partial thromboplastin time; LSIMS, liquid secondary ionisation mass spectrometry; ESI-MS/MS, electrospray ionisation tandem mass spectrometry; PCR, polymerase chain reaction; GC-MS, gas chromatography-mass spectrometry; BSEP, bile acid export pump; FXR, farnesoid X receptor

### Patient MS

This is the Sardinian girl described previously.<sup>6</sup> She was the second child of healthy parents who were not knowingly consanguineous. She presented at three weeks with hyperbilirubinaemia (316  $\mu\text{M}$ , conjugated 145), raised transaminases (aspartate aminotransferase (AST) 2279 U/l; alanine aminotransferase (ALT) 1123 U/l), and a prolonged prothrombin time (15.4 seconds; control 12). Cholestasis persisted and was associated with steatorrhoea, failure to thrive, and rickets. A liver biopsy at three months showed lobular disarray resulting from extensive giant cell transformation and necrotic foci with granulocyte accumulation. Hepatocytes contained fat and bile pigment. Failure to thrive, steatorrhoea, and fat soluble vitamin malabsorption continued despite ursodeoxycholic acid treatment. At eight months her bilirubin was still elevated (88  $\mu\text{M}$ ; conjugated 35  $\mu\text{M}$ ), as were her transaminases (AST 511 U/l, ALT 252 U/l) but  $\gamma$ -glutamyl transpeptidase ( $\gamma$ -GT) was normal (36 U/l). Clotting times were normal but vitamin E was low at 4.8  $\mu\text{M}$  (normal 11.5–35) despite oral supplements. The liver still showed a giant cell hepatitis with steatosis. Treatment with 8 mg/kg/day of chenodeoxycholic acid and 8 mg/kg/day of cholic acid led to normalisation of liver function within three months and she remains well with normal liver function tests at the age of nine years (on bile acid replacement therapy).

### Patient BH

BH is the first child of healthy Pakistani parents who are first cousins. On the first day of life he became jaundiced with dark urine and yellow stools. Cholestatic jaundice persisted and at the age of eight weeks he was found to have a liver edge palpable 3 cm from the right costal margin and a spleen tip palpable 1 cm from the left costal margin. Total bilirubin was 160  $\mu\text{mol/l}$  (conjugated 82  $\mu\text{mol/l}$ ); ALT 333 U/l (normal 5–45);  $\gamma$ -GT normal; alkaline phosphatase (ALP) 842 U/l (65–265); albumin 35 g/l; cholesterol 4.6 mM; vitamin A 97  $\mu\text{g/l}$  (200–430); 25-hydroxy-vitamin D3 15 nmol/l (15–100); vitamin E 8.1  $\mu\text{mol/l}$  (11.5–35); prothrombin time 28 seconds (12–16); activated partial thromboplastin time (APTT) 75 seconds (29–40); and thrombin time 14.1 seconds (8–12). Abdominal ultrasound showed normal hepatic echotexture and a spleen of 6.4 cm (<6.0); the gall bladder could not be seen. A HIDA scan showed no excretion of isotope into the gut. A liver biopsy showed preserved architecture. Portal tracts showed bile duct proliferation but no cholangiolar bile plugging. The parenchymal cells showed marked giant cell transformation with intracellular bile pigment accumulation and occasional single necrotic liver cells. The appearances were of a giant cell hepatitis but there was also marked extramedullary haemopoiesis. Investigations for known causes of hepatitis of infancy were negative except for analysis of plasma and urine bile acids.

BH was treated with chenodeoxycholic acid (8 mg/kg/day) and cholic acid (8 mg/kg/day). Prior to starting treatment, his bilirubin was 446  $\mu\text{mol/l}$ , albumin 31 g/l, ALT 1702 U/l,  $\gamma$ -GT 103 U/l (normal 20–155), and prothrombin time (post parenteral vitamin K) 19 seconds. After six weeks of treatment, values were bilirubin 117, albumin 37, ALT 184,  $\gamma$ -GT 243 (10–100), and prothrombin time 13 seconds. At this point the infant had obvious pruritus and a plasma chenodeoxycholic acid concentration of 128  $\mu\text{mol/l}$  (0.2–12.7). Chenodeoxycholic acid was stopped. Liver function tests started to deteriorate and this continued despite reintroduction of chenodeoxycholic acid. The infant had a lymphocytosis and a positive polymerase chain reaction (PCR) test (but negative IgM and immunofluorescent antigen tests) for cytomegalovirus. Further acute deterioration (prothrombin ratio 2.1) led to referral for liver

transplantation. This was undertaken successfully and the patient is well at the age of 2 years 10 months.

### Patient RM

RM was the second child of Sri Lankan parents who are first cousins. Their first child died at four months from asphyxiating thoracic dystrophy plus cystic malformation of both kidneys. RM was born at 43 weeks gestation and was breast fed. She was treated with phototherapy from day 3 to day 6 because of neonatal jaundice and she presented with jaundice again at two weeks, this time with pale stools and dark urine. Examination at four weeks revealed no hepatomegaly or splenomegaly. Blood tests showed a total bilirubin of 195  $\mu\text{mol/l}$  (conjugated 155); AST 680 U/l; ALT 559 U/l; ALP 818 U/l;  $\gamma$ -GT 29 U/l (normal); lactate dehydrogenase 1496 U/l; and cholesterol 6 mM (1.2–4.6). Prothrombin time and APTT were immeasurably prolonged but corrected almost completely with parenteral vitamin K.

Blood and urine tests for causes of neonatal hepatitis were negative apart from the bile acid abnormalities described below. Ultrasound of the abdomen showed a normal sized liver and spleen, a small gall bladder, and some irregularity in liver density. A liver biopsy revealed a giant cell hepatitis, no increase in bile ducts, and no signs of copper or iron accumulation. Increased extramedullary haematopoiesis was noted.

By the age of six weeks, liver function had deteriorated significantly (international normalised ratio 2.0, APTT >190 seconds, bilirubin 365  $\mu\text{mol/l}$ , ALT 785). She was treated with ursodeoxycholic acid (30 mg twice daily). Bilirubin fell slightly, to 207  $\mu\text{mol/l}$  at 10 weeks. From 10 weeks, RM was treated with chenodeoxycholic acid (10 mg three times daily). There was no improvement in liver function tests and the development of liver failure led to referral for transplantation at 19 weeks. One day after transplantation the patient developed severe cerebral oedema with absence of all reflexes and one day later she died.

### Plasma and urine bile acid analyses

Urine samples from MS and BH were analysed by liquid secondary ionisation mass spectrometry (LSIMS), as described previously.<sup>6,7</sup> A urine and plasma sample from RM were analysed by electrospray ionisation tandem mass spectrometry (ESI-MS/MS) using a “parents of  $m/z$  74” scan for glycine conjugates and a “parents of  $m/z$  80” scan for taurine conjugates.<sup>8</sup> Analyses of (non-sulphated) plasma and urine bile acids by gas chromatography-mass spectrometry (GC-MS) were undertaken using established methods.<sup>6,7,9</sup>

### Analysis of the 5 $\beta$ -reductase gene (*SRD5B1*)

Initial genetic studies of 5 $\beta$ -reductase focused on the characterisation of cDNA from liver biopsy samples.<sup>10</sup> Subsequent to the determination of the genomic sequence of *SRD5B1*,<sup>11</sup> studies concentrated on genomic DNA: the nine exons of the gene from each patient were amplified using a single round of PCR utilising a Gene Amp PCR System 9700 machine with Bioline  $T_{aq}$  polymerase. The temperature program included an initial denaturing step of 94°C for four minutes followed by 28 cycles of denaturing at 94°C for one minute, annealing at 60°C for one minute 30 seconds, and extension at 72°C for two minutes 15 seconds. A final extension step of 72°C for seven minutes was also employed. Details of the primer pairs used are given in table 1. Direct sequencing of exon amplimers was achieved using an ABI Prism 377 automated DNA sequencer with ABI Prism Dye Terminator Cycle Sequencing reactions. Once putative mutations were found, parents and control populations were screened for these mutations using restriction enzyme digests. *BanI* was used to screen the exon 4 amplimer for

**Table 1** Primer pairs for each exon of the 5 $\beta$ -reductase gene and the primer used to generate an amplicon suitable for restriction enzyme digest in patient MS

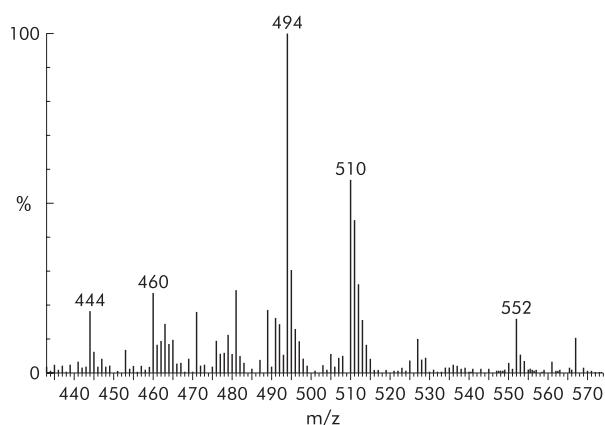
Exon 1	Forward	gggtgtgtaatcctccctccc
	Backward	aagaaacaacaagcaagcaag
Exon 2	Forward	ttctctttcccacaacc
	Backward	cacacacacacacaggaag
Exon 3	Forward	gggctgcttattgtaaaaa
	Backward	acctgggcatctgaactc
Exon 4	Forward	gctcaaatatgaagactggg
	Backward	tgaagtaaaagggtgcatcttc
Exon 5	Forward	ggctgtttgtgacctgtg
	Backward	tcatgagccttctgactcc
Exon 6	Forward	ttgtgctgctctcaatg
	Backward	accaaaggcattccaatca
Exon 7	Forward	gtgactgggtgaatgggaag
	Backward	ttcaatctccaaagcaattc
Exon 8	Forward	tgatccaagcttgaccttc
	Backward	gactacgttctagctagc
Exon 9	Forward	tctgtgggggaattgtttg
	Backward	tgagtcaagctattacactgac
MS RE product	Forward	tgftcttgaatcatttaaggaagc
	Backward	ccittggggaccagtaggaatc

the mutation found in BH, while *DdeI* was used to screen the exon 3 amplicon for the mutation found in RM. *FokI* was used to screen a novel amplicon (see table 1) for the mutation found in MS.

## RESULTS

### Plasma and urine bile acid analyses

Analysis of urine by LSIMS or ESI-MS/MS showed that, in all three patients, the largest bile acid peaks were consistent with a monohydroxy-oxo-cholenoic acid present as the glycine conjugate (m/z 444) and the taurine conjugate (m/z 494) and a dihydroxy-oxo-cholenoic acid present as the glycine conjugate (m/z 460) and the taurine conjugate (m/z 510) (fig 1). Peaks attributable to the glycine and taurine conjugates of chenodeoxycholic acid and cholic acid (m/z 448, 464, 498, and 514) were very small or undetectable above background. The sample from BH showed an additional prominent peak of mass/charge ratio 552, consistent with a taurine conjugated dihydroxy-oxo-cholestenic acid (fig 1). Analysis of plasma and urine samples from MS



**Figure 1** Negative ion liquid secondary ionisation mass spectrometry (LSIMS) analysis of a urine sample from patient BH. Identities of peaks (confirmed by gas chromatography-mass spectrometry): m/z 444 and 460, glycine conjugates of 7 $\alpha$ -hydroxy-3-oxo-4-cholenoic acid and 7 $\alpha$ ,12 $\alpha$ -dihydroxy-3-oxo-4-cholenoic acid; m/z 494, 510, and 552, taurine conjugates of 7 $\alpha$ -hydroxy-3-oxo-4-cholenoic acid, 7 $\alpha$ ,12 $\alpha$ -dihydroxy-3-oxo-4-cholenoic acid, and 7 $\alpha$ ,12 $\alpha$ -dihydroxy-3-oxo-4-cholestenic acid.

and BH by GC-MS following treatment with cholyglycine hydrolase confirmed that the major bile acids were 7 $\alpha$ -hydroxy-3-oxo-4-cholenoic acid and 7 $\alpha$ ,12 $\alpha$ -dihydroxy-3-oxo-4-cholenoic acid (table 2). Chenodeoxycholic acid was undetectable in both plasma samples; cholic acid was absent from the plasma of MS and present in a trace amount in the plasma of BH. Analysis of plasma from MR by ESI-MS/MS indicated that mono- and di-hydroxy-oxocholenoic acids were the major bile acids; chenodeoxycholic acid and cholic acid concentrations were normal/low. (The true concentrations may have been lower than those shown in table 2; there is the potential for isomeric compounds such as *allo* bile acids to interfere with analysis of chenodeoxycholic acid and cholic acid by ESI-MS/MS.)

### Analysis of the 5 $\beta$ -reductase gene (*SRD5B1*)

A point mutation or single base deletion was found in all three patients. The results of the sequencing and restriction enzyme digests are summarised in table 3. All three patients were homozygous for their mutation and all parents were heterozygotes, as was an unaffected sibling of BH. The point mutation found in MS (662 C>T) was not found in a control population of 100 chromosomes; 38 of these were from the Sardinian population and the remaining 62 were unspecified Caucasian samples. The mutations found in patients BH (511 delT) and RM (385 C>T) and were not found in 100 chromosomes from individuals from the Indian subcontinent.

## DISCUSSION

Analysis of bile acids and alcohols in plasma and urine has become an essential part of the investigation of infants with cholestatic jaundice. Several characteristic cholanoic profiles have led to the discovery of mutations in genes encoding enzymes in the bile acid synthesis pathways. These include inborn errors affecting 3 $\beta$ -hydroxy- $\Delta^5$ -C<sub>27</sub>-steroid dehydrogenase/isomerase,<sup>12,13</sup> oxysterol 7 $\alpha$ -hydroxylase,<sup>14</sup> sterol 27-hydroxylase,<sup>15,16</sup> and  $\alpha$ -methyl-acyl-CoA racemase.<sup>17,18</sup> Cholanoic analyses have also helped to define gene defects that affect peroxisome biogenesis<sup>7,9</sup> or peroxisomal  $\beta$ -oxidation enzymes,<sup>19-21</sup> in most of these disorders neurological symptoms dominate the clinical picture.<sup>22,23</sup>

To date, it has proved difficult to establish that infants with cholestatic liver disease and a cholanoic profile characterised by excretion of 3-oxo- $\Delta^4$  bile acids have a genetic deficiency of  $\Delta^4$ -3-oxosteroid 5 $\beta$ -reductase. In some infants, the liver biopsy showed absence of immunoreactive 5 $\beta$ -reductase protein or a truncated protein and this was taken as evidence of a genetic deficiency.<sup>3</sup> However, the possibility of a labile mRNA or protein could not be excluded.

Determination of the human 5 $\beta$ -reductase cDNA sequence<sup>4</sup> allowed Sumazaki *et al* to show that, in at least one infant with liver failure, the 5 $\beta$ -reductase mRNA had a normal sequence.<sup>5</sup> This confirmed the suspicion that some children who excrete 3-oxo- $\Delta^4$  bile acids as the major urinary bile acids do not have genetic 5 $\beta$ -reductase deficiency.

Patient MS had cholanoic profiles that showed almost complete absence of chenodeoxycholic acid and cholic acid. Her liver disease resolved completely on treatment with chenodeoxycholic acid plus cholic acid having failed to respond to ursodeoxycholic acid. This suggested strongly that she had mutations in the 5 $\beta$ -reductase gene. Sequencing of her genomic DNA showed that she was homozygous for a single base substitution 662 C>T. The base substitution abolishes the 5 base recognition sequence for the restriction enzyme *FokI*. Restriction fragment length analyses showed that the patient's parents were heterozygous for the mutation and that it could not be detected in 100 ethnically matched genomic DNA samples, excluding the possibility that it was a

**Table 2** Plasma concentrations of chenodeoxycholic acid and cholic acid and their 3-oxo- $\Delta^4$  analogues in the plasma of children with mutations in the *SRD5B1* gene causing  $\Delta^4$ -3-oxosteroid 5 $\beta$ -reductase deficiency

Bile acid	Plasma concentration ( $\mu\text{mol/l}$ )				
	Patient MS* (age 8 months)	Patient BH* (age 2.5 months)	Patient RM† (age 4 weeks)	Normal infants	Infants with cholestasis
Chenodeoxycholic acid	<0.05	<0.05	0.9	0.2–12.7	13.4–181
Cholic acid	<0.05	0.26	4.0	0.4–6.7	4.7–403
7 $\alpha$ -hydroxy-3-oxo-4-cholenoic acid	1.9	6.7	2.1	<0.05	0–6.5
7 $\alpha$ , 12 $\alpha$ -dihydroxy-3-oxo-4-cholenoic acid	2.1	7.6	2.9	<0.05	0–4.4

\*Measured by gas chromatography-mass spectrometry following enzymatic deconjugation.

†Measured by electrospray ionisation tandem mass spectrometry—may overestimate concentrations of chenodeoxycholic acid and cholic acid because of interference from isomers.

common polymorphism in Caucasians. The 662 C>T mutation is a missense mutation that leads to the amino acid substitution Pro198Leu in the 5 $\beta$ -reductase protein. Comparison of the predicted secondary structure of 5 $\beta$ -reductase with two other NADPH binding proteins, glutathione reductase and NADPH-cytochrome P-450 reductase, suggests that proline 198 is in the NADPH binding domain of the 5 $\beta$ -reductase protein.<sup>4</sup> Substitution of a leucine residue for the proline residue can be predicted to alter the secondary structure of this domain. Thus even if the protein is translated normally and escapes quality control processes, the mutant enzyme is unlikely to bind NADPH normally and is therefore likely to be catalytically inactive.

Patient BH, like MS, had cholanoic profiles that showed almost undetectable amounts of chenodeoxycholic acid and cholic acid. He also showed an initial response to treatment with chenodeoxycholic acid and cholic acid, although a subsequent deterioration, perhaps due to cytomegalovirus infection, meant that he needed a liver transplant. Again, the clinical and biochemical features strongly suggested mutations in the 5 $\beta$ -reductase gene. Analysis of genomic DNA showed that he was homozygous for a mutation, 511delT, that produces a premature stop codon in exon 5. This mutation can be predicted to produce a truncated, catalytically inactive protein. Analysis of genomic DNA from BH's parents and his unaffected sibling showed that they were heterozygous for the 511delT mutation.

Patient RM also had a urinary cholanoic profile (showing m/z 444>>448, 460>>464, 494>>498, and 510>>514) that suggested a major block in the synthesis of chenodeoxycholic acid and cholic acid, compatible with mutations in the 5 $\beta$ -reductase gene. Analysis of her plasma by ESI-MS suggested that some synthesis of chenodeoxycholic acid and cholic acid may be occurring although the concentrations were still low for an infant with cholestatic jaundice. Treatment with bile acids was only started when her liver disease was quite advanced. She showed slight improvement in her cholestasis with ursodeoxycholic acid but no further improvement with chenodeoxycholic acid. Nevertheless, she too seemed a good candidate for genetic 5 $\beta$ -reductase deficiency; we knew from

our experience with BH that this disorder could progress to liver failure in infancy. Analysis of genomic DNA showed that she was homozygous for a missense mutation (385 C>T). The Leu106Phe substitution occurs within the alpha-3 helix of the 5 $\beta$ -reductase enzyme. Leucine 106 is conserved within 41 of the 42 members of the aldo keto-reductase super family (98%) according to the alignments published by Jez and colleagues.<sup>24</sup> The missense mutation in RM might produce 5 $\beta$ -reductase enzyme with a little residual activity. This would explain why the plasma concentrations of chenodeoxycholic acid and cholic acid were apparently higher than in the two other patients.

Detection of *SRD5B1* mutations in these three patients allows us to define the effects of genetic 5 $\beta$ -reductase deficiency. All three patients had cholestatic liver disease of neonatal onset; liver function tests showed a normal  $\gamma$ -GT at a time when transaminases were considerably elevated. This phenomenon, as well as evidence of malabsorption of fat and fat soluble vitamins, is seen in other defects of bile acid synthesis (for example, 3 $\beta$ -hydroxysteroid- $\Delta^5$ -C<sub>27</sub>-steroid dehydrogenase deficiency) as well as in defects of bile acid secretion (for example, progressive familial intrahepatic cholestasis type II due to mutations in the gene encoding the bile acid export pump (*BSEP*)). The reason for the low  $\gamma$ -GT in these patients is not certain. It is known that  $\gamma$ -GT is normally located on microvilli at the canalicular surface of hepatocytes and bile duct epithelial cells. It is probably only released from the microvilli when bile acids are secreted into the canaliculi and exert a detergent effect on the membranes lining the biliary system. Thus if bile acids are not entering the canaliculi there is reduced production of the soluble form of  $\gamma$ -GT that normally accumulates in the circulation in response to damage to bile ducts and hepatocytes.

The bile acid profiles in all three patients with *SRD5B1* mutations showed amounts of chenodeoxycholic acid and cholic acid that were very low for an infant with cholestasis; this is consistent with the notion that  $\Delta^4$ -3-oxosteroid 5 $\beta$ -reductase is required for all biosynthetic pathways for the major primary bile acids. Failure to synthesise chenodeoxycholic acid and cholic acid may lead to low hepatocyte

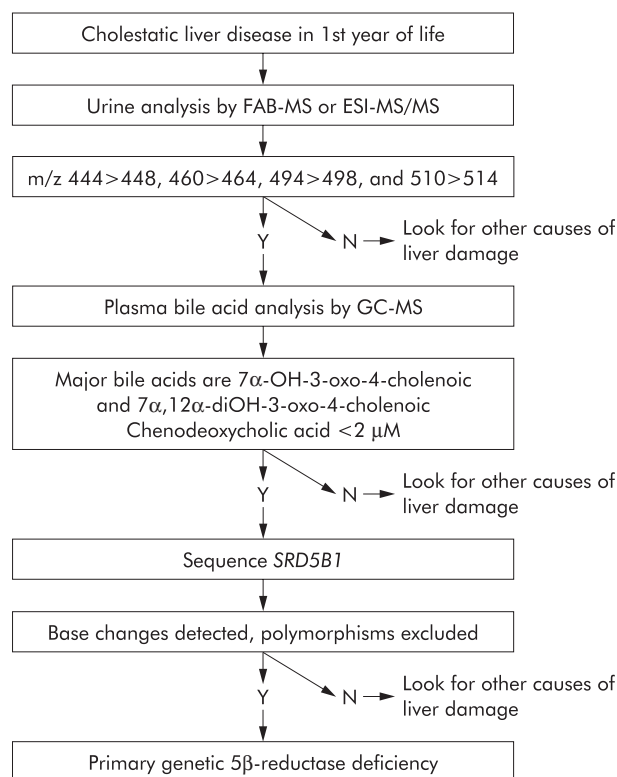
**Table 3** Summary of the mutations found on DNA sequencing of the 5 $\beta$ -reductase gene and subsequent restriction digest of parental/sibling DNA

Patient	Sequencing	Restriction enzyme digest
MS	662 C>T missense mutation causing a Pro198Leu amino acid substitution	Parents heterozygous for the 662 C>T missense mutation
BH	511T deletion causing frame shift and a premature stop codon in exon 5	Parents and unaffected sibling heterozygous for the 511T deletion
RM	385 C>T missense mutation causing a Leu106Phe amino acid substitution	Parents heterozygous for the 385 C>T missense mutation

concentrations of these bile acids with consequent failure to activate the farnesoid X receptor (FXR) and hence switch on the bile acid export pump (BSEP) and the canalicular conjugated bilirubin transporter (MRP2).<sup>25</sup> Failure to activate MRP2 may contribute to the jaundice in these patients and also to poor excretion of HIDA.

There are some significant differences between the biochemical findings in these three patients and other putative cases of 5 $\beta$ -reductase deficiency. Thus the cases reported by Setchell and colleagues<sup>2</sup> and Kimura and colleagues<sup>26,27</sup> had elevated plasma concentrations of chenodeoxycholic acid and a high ratio of chenodeoxycholic acid to cholic acid. Other cases reported by Kimura *et al* had increased concentrations of total plasma bile acids (method of assay and chenodeoxycholic acid/cholic acid ratio not specified).<sup>28</sup> Our own experience is that many infants with severe liver disease (and some infants with milder disease) show this pattern of high plasma chenodeoxycholic acid, high cheno/cholic ratio, and moderate 3-oxo $\Delta^4$  bile aciduria—it is much commoner than the profiles described above for patients with proven *SRD5B1* mutations. There are four possible explanations for a cholanoic profile characterised by increased excretion of 3-oxo- $\Delta^4$  bile acids in urine and a high plasma chenodeoxycholic acid concentration.

- These patients have *SRD5B1* mutations that leave some residual 5 $\beta$ -reductase activity. Some chenodeoxycholic acid is synthesised and inhibition of BSEP by 3-oxo- $\Delta^4$  bile acids<sup>29</sup> causes reflux of bile acids into the plasma leading to elevated plasma concentrations.
- These patients do not have a genetic defect in the *SRD5B1* gene but rather secondary changes in the liver lead to inactivation of the 5 $\beta$ -reductase enzyme. The 5 $\beta$ -reductase protein may be unstable; certainly early attempts at purification proved problematical.<sup>30</sup> It may be prone to chemical attack. Zhu *et al* showed that it can form adducts with acetaldehyde *in vivo*.<sup>31</sup>
- There are no mutations in the *SRD5B1* gene but the amount of mRNA in liver cells is reduced. mRNA may be unstable. Kondo *et al* showed that the 3' untranslated region of the 5 $\beta$ -reductase mRNA contained multiple AU rich elements.<sup>4</sup> Such AUREs are associated with rapidly degrading mRNAs such as those encoding cytokines and lymphokines.<sup>32</sup> It is also possible that expression of *SRD5B1* is regulated by transcription factors as is the case with other key enzymes in oxysterol metabolism and bile acid synthesis (see below).
- These patients have persistence of, or reversion to, the fetal pattern of bile acid metabolism. The major pathway of bile acid synthesis in the fetus probably starts with the production of 27-hydroxycholesterol and produces mainly chenodeoxycholic acid.<sup>14</sup> This is the predominant bile acid in the fetus; however, significant amounts of 7 $\alpha$ -hydroxy-3-oxo-4-cholenoic acid are also produced.<sup>33</sup> BSEP is probably inactive in fetal life<sup>34</sup> and it is therefore likely that a substantial proportion of the chenodeoxycholic acid (and 7 $\alpha$ -hydroxy-3-oxo-4-cholenoic acid) synthesised in the hepatocyte enter the blood stream. Increased cholic acid synthesis and activation of the BSEP normally occur at the time of birth; if this activation does not occur and the fetal pattern of bile acid metabolism persists, one would predict high plasma chenodeoxycholic acid, high cheno/cholic ratio, and 7 $\alpha$ -hydroxy-3-oxo-4-cholenoic acid in plasma. It is becoming clear that regulation of bile acid synthesis and secretion is controlled by nuclear receptors such as FXR, and to a lesser extent by the liver X receptors and the pregnane X receptor/steroid and xenobiotic receptor.<sup>25,35</sup> Recent studies suggest that full activation of the BSEP might require the natural analogue



**Figure 2** Suggested algorithm for distinguishing between primary genetic 5 $\beta$ -reductase deficiency and other cause of 3-oxo- $\Delta^4$  bile aciduria. ESI-MS/MS, electrospray ionisation tandem mass spectrometry; GC-MS, gas chromatography-mass spectrometry; FAB-MS, fast atom bombardment-mass spectrometry.

of guggulsterone as well as chenodeoxycholic acid (a known agonist at the FXR receptor).<sup>36</sup> It is possible therefore that persistence of a fetal pattern of bile acid synthesis and secretion is caused by failure of activation of transcription factors (including FXR) by the natural analogue of guggulsterone.

Further work is required to establish whether patients with 3-oxo- $\Delta^4$  bile aciduria and elevated plasma chenodeoxycholic acid concentrations have mutations in the *SRD5B1* gene or whether they have other gene defects or non-genetic liver diseases that lead to secondary reduction in 5 $\beta$ -reductase enzyme activity. This is of more than academic interest. Treatment with chenodeoxycholic acid and cholic acid was extremely effective in one of our patients with *SRD5B1* mutations (the only one for whom treatment was started when bilirubin was <200  $\mu$ M, ALT was <600 U/l, and the post vitamin K prothrombin time was normal). In patients with high plasma chenodeoxycholic acid concentrations, the combination of ursodeoxycholic acid and cholic acid appeared to be more effective.<sup>2</sup> In the future, nuclear receptor agonists may have a role in carefully defined patients. Based on our current knowledge of 5 $\beta$ -reductase mutations, a suggested algorithm for diagnosis of genetic 5 $\beta$ -reductase deficiency is shown in fig 2.

## ACKNOWLEDGEMENTS

This work was funded by grants from the Engineering and Physical Sciences Research Council (research studentship for HAL), Child Health Research Appeal Trust, and Wellcome Trust (equipment grant). We would like to thank Dr Giuseppe Novelli (Universita di Roma) and Professor Steve Humphries, UCL, for supplying ethnically matched DNA control samples.

.....

### Authors' affiliations

**H A Lomonde, P T Clayton**, Biochemistry, Endocrinology, and Metabolism Unit, Institute of Child Health, University College London with Great Ormond Street Hospital for Children NHS Trust, London, UK  
**E J Custard, P J Scambler**, Molecular Medicine Unit, Institute of Child Health, University College London with Great Ormond Street Hospital for Children NHS Trust, London, UK  
**J Bouquet**, Erasmus MC/Sophia Children's Hospital, Rotterdam, the Netherlands  
**M Duran, H Overmars**, Laboratory for Genetic Metabolic Diseases, Academic Medical Centre, Amsterdam, the Netherlands

### REFERENCES

- 1 Clayton PT, Patel E, Lawson AM, *et al.* 3-Oxo-delta 4 bile acids in liver disease. *Lancet* 1988;**1**:1283-4.
- 2 Setchell KD, Suchy FJ, Welsh MB, *et al.* Delta 4-3-oxosteroid 5 beta-reductase deficiency described in identical twins with neonatal hepatitis. A new inborn error in bile acid synthesis. *J Clin Invest* 1988;**82**:2148-57.
- 3 Schneider BL, Setchell KD, Whittington PF, *et al.* Delta 4-3-oxosteroid 5 beta-reductase deficiency causing neonatal liver failure and hemochromatosis. *J Pediatr* 1994;**124**:234-8.
- 4 Kondo KH, Kai MH, Setoguchi Y, *et al.* Cloning and expression of cDNA of human delta 4-3-oxosteroid 5 beta-reductase and substrate specificity of the expressed enzyme. *Eur J Biochem* 1994;**219**:357-63.
- 5 Sumazaki R, Nakamura N, Shoda J, *et al.* Gene analysis in  $\Delta^4$ -3-oxosteroid 5 $\beta$ -reductase deficiency. *Lancet* 1997;**349**:329.
- 6 Clayton PT, Mills KA, Johnson AW, *et al.* Delta 4-3-oxosteroid 5 beta-reductase deficiency: failure of ursodeoxycholic acid treatment and response to chenodeoxycholic acid plus cholic acid. *Gut* 1996;**38**:623-8.
- 7 Lawson AM, Madigan MJ, Shortland D, *et al.* Rapid diagnosis of Zellweger syndrome and infantile Refsum's disease by fast atom bombardment-mass spectrometry of urine bile salts. *Clin Chim Acta* 1986;**161**:221-31.
- 8 Mills K, Mushtaq I, Johnson A, *et al.* A method for the quantitation of conjugated bile acids in dried blood spots using electrospray ionisation-mass spectrometry. *Pediatr Res* 1998;**43**:361-8.
- 9 Clayton PT, Lake BD, Hall NA, *et al.* Plasma bile acids in patients with peroxisomal dysfunction syndromes; analysis by capillary gas chromatography-mass spectrometry. *Eur J Pediatr* 1987;**146**:166-73.
- 10 Lomonde HA. A study using tandem mass spectrometry and molecular biological techniques to facilitate the diagnosis of inborn errors of bile acid synthesis, with particular reference to  $\Delta^4$ -3-oxosteroid 5 $\beta$ -reductase deficiency, PhD thesis. London: University of London, 2000.
- 11 Charbonneau A, The VL. Genomic organization of a human 5beta-reductase and its pseudogene and substrate selectivity of the expressed enzyme. *Biochim Biophys Acta* 2001;**1517**:228-35.
- 12 Clayton PT, Leonard JV, Lawson AM, *et al.* Familial giant cell hepatitis associated with synthesis of 3 $\beta$ ,7 $\alpha$ -dihydroxy- and 3 $\beta$ ,7 $\alpha$ ,12 $\alpha$ -trihydroxy-5-cholenic acids. *J Clin Invest* 1987;**79**:1031-8.
- 13 Schwarz M, Wright AC, Davis DL, *et al.* The bile acid synthetic gene 3 beta-hydroxy-Delta(5)-C-27-steroid oxidoreductase is mutated in progressive intrahepatic cholestasis. *J Clin Invest* 2000;**106**:1175-84.
- 14 Setchell KD, Schwarz M, O'Connell NC, *et al.* Identification of a new inborn error in bile acid synthesis: mutation of the oxysterol 7alpha-hydroxylase gene causes severe neonatal liver disease. *J Clin Invest* 1998;**102**:1690-703.
- 15 Clayton PT, Casteels M, Mieli-Vergani G, *et al.* Familial giant cell hepatitis with low bile acid concentrations and increased urinary excretion of specific bile alcohols: a new inborn error of bile acid synthesis? *Pediatr Res* 1995;**37**:424-31.
- 16 Clayton PT, Verrips A, Sijm AM, *et al.* Mutations in the sterol 27-hydroxylase gene (*CYP27A*) cause hepatitis of infancy as well as cerebrotendinous xanthomatosis. *J Inher Metab Dis* 2002;**25**:501-13.
- 17 Van Veldhoven PP, Meyhi E, Squires RH, *et al.* Fibroblast studies documenting a case of peroxisomal 2-methylacyl-CoA racemase deficiency: possible link between racemase deficiency and malabsorption and vitamin K deficiency. *Eur J Clin Invest* 2001;**31**:714-22.
- 18 Setchell KDR, Heubi JE, Bove KE, *et al.* Liver disease caused by failure to racemize trihydroxycholestanic acid: Gene mutation and effect of bile acid therapy. *Gastroenterology* 2003;**124**:217-32.
- 19 Clayton PT, Patel E, Lawson AM, *et al.* Bile acid profiles in peroxisomal 3-oxoacyl-CoA thiolase deficiency. *J Clin Invest* 1990;**85**:1267-73.
- 20 van Grunsven I, van Berkel E, Ijlst L, *et al.* Peroxisomal D-hydroxyacyl-CoA dehydrogenase deficiency, a new peroxisomal disorder: resolution of the enzyme defect and its molecular basis. *Proc Natl Acad Sci U S A* 1998;**95**:2128-33.
- 21 Ferdinandusse S, van Grunsven EG, Oostheim W, *et al.* Reinvestigation of peroxisomal 3-ketoacyl-CoA thiolase deficiency: identification of the true defect at the level of d-bifunctional protein. *Am J Hum Genet* 2002;**70**:1589-93.
- 22 Gould SJ, Raymond GV, Valle D. The peroxisome biogenesis disorders. In: Scriver CR, Beaudet MD, Sly WS, *et al.*, eds. *The metabolic and molecular bases of metabolic disease*, 8th edn. New York: McGraw Hill, 2001:3181-218.
- 23 Clayton PT. Clinical consequences of disorders of peroxisomal  $\beta$ -oxidation. *Biochem Soc Trans* 2001;**29**:298-305.
- 24 Jez JM, Bennett MJ, Schlegel BP, *et al.* Comparative anatomy of the aldo-keto reductase superfamily. *Biochem J* 1997;**326**:625-36.
- 25 Edwards PA, Kast HR, Anisfeld AM. BAREing it all: the adoption of LXR and FXR and their role in lipid homeostasis. *J Lipid Res* 2002;**43**:2-12.
- 26 Kimura A, Yuge K, Yukizane S, *et al.* Abnormal low ratio of cholic acid to chenodeoxycholic acid in a cholestatic infant with severe hypoglycemia. *J Pediatr Gastroenterol Nutr* 1991;**12**:383-7.
- 27 Kimura A, Nittono H, Takei H, *et al.* Abnormally low ratio of cholic acid to chenodeoxycholic acid due to a deficiency of 3-oxo-delta4-steroid 5beta-reductase. *Pediatr Int* 2000;**42**:594.
- 28 Kimura A, Suzuki M, Tohma M, *et al.* Increased urinary excretion of 3-oxo-delta4 bile acids in Japanese patients with idiopathic neonatal cholestasis. *J Pediatr Gastroenterol Nutr* 1998;**27**:606-9.
- 29 Stieger B, Zhang J, O'Neill B, *et al.* Differential interaction of bile acids from patients with inborn errors of bile acid synthesis with hepatocellular bile acid transporters. *Eur J Biochem* 1997;**244**:39-44.
- 30 Berseus O. Conversion of cholesterol to bile acids in rat: purification and properties of a delta-4-3-ketosteroid 5-beta-reductase and a 3-alpha-hydroxysteroid dehydrogenase. *Eur J Biochem* 1967;**2**:493-502.
- 31 Zhu Y, Fillenwarth MJ, Crabb D, *et al.* Identification of the 37-kd rat liver protein that forms an acetaldehyde adduct in vivo as delta 4-3-ketosteroid 5 beta-reductase. *Hepatology* 1996;**23**:115-22.
- 32 Ross J. Control of messenger RNA stability in higher eukaryotes. *Trends Genet* 1996;**12**:171-5.
- 33 Setchell KDR, Dumaswala R, Colombo C, *et al.* Hepatic bile acid metabolism during early development revealed from the analysis of human fetal gallbladder bile. *J Biol Chem* 1988;**263**:16637-44.
- 34 Kinchuk VS, Okada T, Akimura K, *et al.* Asynchronous expression and colocalisation of Bsep and Mrp2 during development of rat liver. *Am J Physiol Gastrointest Liver Physiol* 2002;**282**:G540-8.
- 35 Francis GA, Fayard E, Picard F, *et al.* Nuclear receptors and the control of metabolism. *Annu Rev Physiol* 2003;**65**:261-311.
- 36 Cui J, Huang L, Zhao A, *et al.* Guggulsterone is an FXR antagonist in coactivator association assays but acts to enhance transcription of bile salt export pump. *J Biol Chem* 2003;**278**:10214-20.