Serum 27-hydroxycholesterol in patients with primary biliary cirrhosis suggests alteration of cholesterol catabolism to bile acids via the acidic pathway

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Abstract **Reduced cholesterol synthesis has been reported in patients with primary biliary cirrhosis but no data are available on changes in cholesterol catabolism induced by the disease. Serum levels of 7α-hydroxycholesterol and 27-hydroxycholesterol have been measured in 25 patients (either normocholesterolemic or hypercholesterolemic) with primary biliary cirrhosis and in control subjects. To evaluate cholesterol synthesis, serum levels of lathosterol were measured, and campesterol and sitosterol were considered to reflect intestinal absorption and biliary elimination of sterols. In normocholesterolemic patients with primary biliary cirrhosis, lathosterol was significantly lower than in normocholesterolemic controls ($P < 0.05$) whereas no difference was found between hypercholesterolemic patients and hypercholesterolemic controls. Serum concentrations of sitosterol were significantly higher in both normocholesterolemic and hypercholesterolemic patients with primary biliary cirrhosis as compared with the respective controls ($P < 0.01$). In patients with primary biliary cirrhosis, serum 7α-hydroxycholesterol was slightly higher than in controls. 27-Hydroxycholesterol was significantly higher in hypercholesterolemic compared to normocholesterolemic controls ($P < 0.05$) and a significant linear correlation ($r = 0.771$; $P < 0.001$) was found between 27-hydroxycholesterol and cholesterol. In contrast, in patients with primary biliary cirrhosis, high cholesterol concentrations were not associated with increased serum levels of 27-hydroxycholesterol. Our data confirm that in patients with primary biliary cirrhosis, cholesterol synthesis and biliary elimination of sterols are impaired and also suggest that both the feedback regulation of retained bile acids on cholesterol 7α-hydroxylase and the scavenger effect on elevated serum cholesterol by cholesterol 27-hydroxylase are deficient in these patients.—**Del Puppo, M., M. Galli Kienle, M. L. Petroni, A. Crosignani, and M. Podda. **Serum 27-hydroxycholesterol in patients with primary biliary cirrhosis suggests alteration of cholesterol catabolism to bile acids via the acidic pathway. *J. Lipid Res.* 1998.** 39: **2477–2482.**

Supplementary key words 27-hydroxycholesterol • 7α-hydroxycholesterol • mass spectrometry • hypercholesterolemia • cholesterol metabolism • lathosterol • dietary sterols • cholesterol transport •bile acid synthesis

Primary biliary cirrhosis (PBC) is a chronic liver disease characterized by severe intrahepatic cholestasis commonly associated with hypercholesterolemia and marked alterations of the enterohepatic circulation of bile acids (i.e., decreased biliary secretion with elevated serum levels of bile acids) (1). Several data have been reported on changes in cholesterol metabolism induced by this cholestatic liver disease (2, 3). Reliable information is available on cholesterol synthesis, while no data have been reported on cholesterol catabolism. Recent studies have demonstrated that serum levels of sterol precursors of cholesterol, which are considered to be indices of cholesterol synthesis (4), and of dietary sterols, which are related to intestinal absorption (5) and to biliary secretion, are modified in these patients compared to controls (3, 6). In particular, it has been reported that lathosterol is lower in PBC patients than in control subjects, probably reflecting decreased cholesterol synthesis, whereas increased plant sterol levels are mainly due to impaired biliary elimination.

Hydroxylation of cholesterol at position 7α is the first and rate-limiting step of the "neutral pathway" leading to bile acid synthesis and the mechanism of its regulation is still controversial. Classically, bile acids returning to the liver through the enterohepatic circulation were considered as the effectors of a feedback control on the biosynthetic pathway (7, 8). Accordingly, a significant decrease in bile acid synthesis was reported in patients with liver disease (9). However, this may reflect an impairment of liver function rather than a feedback regulation and the role of cholestasis remains to be established, especially in view of the surprising observation that in experimental

Abbreviations: PBC, primary biliary cirrhosis; NC, normocholesterolemic controls, HC, hypercholesterolemic controls; NC-PBC, normocholesterolemic patients with PBC; HC-PBC, hypercholesterolemic patients with PBC; UDCA, ursodeoxycholic acid; BHT, 2,6-di-tert-butyl-4-methyl-phenol; TMS, trimethylsilylethers; TSIM-PIP, trimethyl silylimidazole:piperidine.

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cholestasis the activity of cholesterol 7α-hydroxylase is even increased (10, 11). Determination of serum levels of 7α-hydroxycholesterol, an indirect index of cholesterol 7α-hydroxylase activity, may be useful for the study of cholesterol catabolism (12) as well as determination of 27-hydroxycholesterol which is related to the activity of sterol 27-hydroxylase catalyzing the first step of an alternative "acidic pathway" leading specifically to the formation of chenodeoxycholic acid (13, 14).

In the present study we have examined several parameters of cholesterol synthesis (lathosterol), absorption and elimination (campesterol and sitosterol), and catabolism (7α-hydroxycholesterol and 27-hydroxycholesterol) in 25 patients with PBC with different stages of the disease and in two groups of control subjects (i.e., 10 normocholesterolemic and 10 hypercholesterolemic subjects).

MATERIALS AND METHODS

Patients and study design

Twenty-five patients were enrolled into the study. Diagnosis of PBC was made according to the usual biochemical, serological, clinical, and histological criteria (1). Ultrasound examination of the upper abdomen was carried out, within the previous 3 months, in order to exclude the presence of liver tumors or extrahepatic biliary obstruction. At entry all patients and controls were put on a standardized 1800 Kcal hypolipidemic diet regimen for at least 4 weeks before blood collection. None of them had been treated with UDCA in the previous 3 months before enrollment.

Individual characteristics of PBC patients are reported in **Table 1**. Twelve patients had increased baseline values of serum cholesterol (HC-PBC), whereas the remaining 13 were normocholesterolemic (NC-PBC). Increased baseline values of serum cholesterol were defined as serum cholesterol concentration over 240 mg/dl. Fourteen patients had liver cirrhosis (7 in the normocholesterolemic group and 7 in the hypercholesterolemic group) and only 4 patients had serum bilirubin concentration higher than 2 mg/dl (2 in the normocholesterolemic group and 2 in the hypercholesterolemic group). These latter four patients had experienced liver cirrhosis: variceal bleeding occurred in one (FP) while hepatic encephalopathy or ascites occurred in all. However, at the time of the study, they were in a rather stable condition.

Two different control groups were selected: *a*) 10 normocholesterolemic controls (NC) consisting of healthy volunteers (8 females, 2 males, mean age 55 ± 10); *b*) 10 hypercholesterolemic controls (HC) enrolled in a series of hyperlipidemic patients (9 females, 1 male, mean age 63 ± 8). Criteria for enrollment in this group were serum cholesterol concentrations >240 mg/dl. All the patients and controls had a body mass index ranging from minus to plus 20% of the ideal value.

After a 4-week run-in period during which all patients and all controls followed the standard diet, blood was collected after an overnight fast. After centrifugation, 2,6-di-tert-butyl-4-methylphenol (BHT) was added as an antioxidant to a final concentration of 50 μg/ml serum. Serum samples were then frozen at −20°C.

Routine automated methods were used to estimate serum concentration of albumin, cholinesterase, bilirubin, and plasma prothrombin. Routine analyses of serum total cholesterol were performed by enzymatic reactions.

Materials

All solvents were obtained from Merck (Darmstadt, Germany) and were of analytical grade. 5α-Cholestane and 19-hydroxycholesterol used as internal standards (IS) and BHT were purchased from Sigma Chemical Co. (St. Louis, MO). Deuterated lathosterol was synthesized (15). Silica cartridge columns (Supelclean

TABLE 1. Clinical characteristics of the 25 patients with PBC enrolled into the study

Patients	Sex	Age	Liver Cirrhosis	Serum Bilirubin	Serum Albumin	Serum Cholesterol	HDL-cholesterol
		yr		*mg/dl*	*g/dl*	*mg/dl*	
AN	F	53	yes	31.0	3.9	448	23
BL	F	69	no	0.5	3.7	215	47
BMA	F	69	no	1.1	4.6	360	149
BC	F	49	yes	0.9	4.0	181	61
BM	F	72	yes	0.6	4.4	264	84
CL	F	56	no	1.0	4.2	215	45
CP	M	77	yes	1.2	4.4	136	54
FD	F	62	yes	0.8	3.9	279	50
FP	M	58	yes	4.2	3.8	349	17
GG	F	61	yes	0.7	3.9	201	75
MC	F	65	yes	0.7	4.3	215	50
ML	F	62	no	0.7	4.2	276	48
NE	F	66	yes	2.2	3.5	124	20
OR	F	54	no	0.5	3.8	199	45
PM	F	65	yes	0.8	4.3	261	95
PS	F	45	yes	3.3	3.3	196	21
PW	F	68	yes	0.5	3.8	246	55
RE	F	58	no	1.0	3.7	230	49
SO	F	62	yes	0.4	4.2	194	50
SL	F	56	no	0.6	4.6	316	148
SA	F	60	no	0.5	4.8	356	52
SE	F	29	no	0.7	3.8	235	57
SG	F	57	yes	0.5	4.5	262	83
TM	F	60	no	0.5	4.3	280	90
TMP	F	57	no	0.4	3.8	179	64

LC-Si, size 1 ml) and trimethylsilylimidazole were obtained from Supelco Inc. (Bellefonte, PA).

Sample preparation for the determination of lathosterol and plant sterols (campesterol and sitosterol)

Deuterated lathosterol and 5α-cholestane were added to 0.1-ml serum samples as solutions of 0.1 μg/μl (10 μl) in ethyl acetate. Alkaline hydrolysis was carried out with 1 ml 1 N NaOH in 90% ethanol at 60°C for 90 min under nitrogen; saline was then added (1 ml), and sterols were extracted with 2 ml of petroleum ether and taken to dryness. Sterols were then converted into trimethylsilylethers (TMS) with 15 μl of trimethylsilylimidazole:piperidine (1:1) (TSIM-PIP) for 10 min at room temperature. Aliquots (1–2 μl) of the silylated mixtures were injected for GC–MS analysis. Results obtained for lathosterol and dietary sterols are expressed as μg/100 mg of total cholesterol.

Sample preparation for the determination of 7α-hydroxycholesterol and 27-hydroxycholesterol

Extraction and purification of hydroxysterols was carried out according to Hahn, Reichel, and von Bergmann (16) using 19-hydroxycholesterol instead of deuterated 7α-hydroxycholesterol as internal standard. Briefly, 19-hydroxycholesterol (250 ng in 10 μl of a solution of 25 μg/ml in ethyl acetate) and 1 ml 1 N NaOH in 90% ethanol were added to 0.2 ml serum and alkaline hydrolysis was performed. After cooling, 1 ml of saline was added and oxysterols were extracted with 2 ml petroleum ether. The organic phase was evaporated to dryness under a stream of nitrogen and purified by solid phase extraction with a silica cartridge column. The latter fraction was evaporated and sterols were converted into trimethylsilylethers with TSIM-PIP as described above.

Conditions for GC-MS analysis

Analysis of sterols was carried out using a Hewlett-Packard 5988 instrument. The spectrometer was set at 70 eV ion energy, 0.1 mA emission current, and 280°C transfer line temperature. Separation of sterols was achieved by an SPB5 (Supelco Inc., Bellefonte, PA) capillary column 0.32 mm i.d., 0.25 μm film thickness, 30 m long, operating at 20 ml/min helium flow rate. Column temperature was programmed from 180° to 300°C at 10°C/min.

Selected Ion Monitoring (SIM) mode was used recording ions at m/z 372 for detection of cholestane, m/z 255 and 259 for lathosterol and deuterated lathosterol, and m/z 382 and 396 for campesterol and sitosterol, m/z 353 for 19-hydroxycholesterol and m/z 456 for 7α-hydroxycholesterol, and 27-hydroxycholesterol.

Calibration curves were prepared by spiking serum with a fixed amount of each internal standard and increasing amounts of the above-mentioned sterols. These samples were treated and analyzed as the experimental samples. Concentrations were calculated on the basis of the slope of the standard curve and on the peak area ratio (sterol/IS) found in the sample. The assay results were linear ($r > 0.98$) in the tested ranges (50–300 ng for lathosterol, 0.1–2 μg for campesterol, 0.1–2 μg for sitosterol, 2–50 ng for 7α-hydroxycholesterol, and 10–100 ng for 27-hydroxycholesterol).

Statistics

Values are reported as mean ± SEM. Significance of the differences was evaluated by the Student's t test. Correlation was checked by linear regression analysis.

RESULTS

Sterols

Serum concentrations of cholesterol, lathosterol, and dietary sterols are reported in **Table 2**. Serum cholesterol ranged from 124 to 235 mg/dl in NC-PBC patients and from 246 to 454 mg/dl in HC-PBC patients. In control subjects, ranges were 142–203 mg/dl and 204–302 for NC and HC groups, respectively. In the latter group, five subjects responded to the diet so that their cholesterol level at the time of blood collection for the evaluation of cholesterol metabolism was lower than 240 mg/dl, while for the other five subjects levels remained over 240 mg/dl.

PBC patients, both NC and HC, had significantly ($P < 0.05$) reduced lathosterol levels compared to NC controls. Lathosterol levels did not differ in PBC patients with liver cirrhosis (63 ± 8 μg/100 mg total cholesterol, n = 14) compared with non-cirrhotic patients (69 ± 6 μg/100 mg

TABLE 2. Total cholesterol, lathosterol and plant sterols in serum of PBC patients and controls

Lipids	NC Controls (n = 10)	HC Controls (n = 10)	NC-PBC (n = 13)	HC-PBC (n = 12)	Total PBC (n = 25)
			mg/dl		
Cholesterol	164 ± 6	248 ± 12 (282 ± 8)[a,**] (214 ± 4)[b]	194 ± 9	300 ± 18**	245 ± 14
			μg/100 mg total cholesterol		
Lathosterol	105 ± 17	70 ± 8 (59 ± 11)[a] (81 ± 10)[b]	67 ± 7*	64 ± 9*	66 ± 5
Campesterol	88 ± 6	134 ± 24 (178 ± 34)[a,**] (89 ± 22)[b]	269 ± 79*	220 ± 52*	245 ± 47
Sitosterol	166 ± 29	186 ± 44 (270 ± 50)[a] (101 ± 51)[b]	549 ± 92** (n = 12)	515 ± 117** (n = 8)	535 ± 70 (n = 20)

Values are mean ± SEM.
[a]Subjects not responding to the diet (see Results), n = 5.
[b]Subjects responding to the diet (see Results), n = 5.
*$P < 0.05$ and **$P < 0.01$ vs. NC controls.

TABLE 3. Oxysterols in serum of PBC patients and controls

Hydroxysterol	NC Controls (n = 9)	HC Controls (n = 9)	NC-PBC (n = 11)	HC-PBC (n = 8)	Total PBC (n = 19)
			μg/dl		
7α-Hydroxycholesterol	5.9 ± 1.6	5.7 ± 1.4 (6.1 ± 2.7)[a] (5.2 ± 0.5)[b]	10 ± 2.6	8.8 ± 1.8	9.5 ± 1.7
27-Hydroxycholesterol	16 ± 2.5	26.6 ± 2.8* (30.5 ± 3.9)[a] (21.7 ± 3.0)[b]	18 ± 1.5	22.2 ± 1.6**	19.8 ± 1.2

Values are mean ± SEM.
[a]Subjects not responding to the diet (see Results), n = 5.
[b]Subjects responding to the diet (see Results), n = 4.
*$P < 0.05$ vs. NC controls.
**$P < 0.05$ vs. HC controls not responding to the diet.

total cholesterol, n = 11). In PBC subjects with increased bilirubin level (>2 mg/dl), lathosterol was significantly lower than in subjects with non-elevated bilirubin (38 ± 9 μg/100 mg total cholesterol vs. 71 ± 5; $P < 0.05$; n = 4 and n = 21, respectively).

In PBC patients, both NC and HC, dietary sterol levels were higher that in control subjects (Table 2). No significant differences were found between patients with and without liver cirrhosis (254 ± 76 μg/100 mg total cholesterol and 235 ± 53 μg/100 mg total cholesterol for campesterol; 573 ± 103 μg/100 mg total cholesterol and 478 ± 88 μg/100 mg total cholesterol for sitosterol; n = 12 and n = 8, respectively). Subjects with increased bilirubin levels had significantly higher values of campesterol (458 ± 245 μg/100 mg total cholesterol vs. 205 ± 31; $P < 0.05$; n = 4 and n = 21, respectively).

Oxysterols

Serum levels of 7α-hydroxycholesterol and 27-hydroxycholesterol are reported in **Table 3**. 7α-Hydroxycholesterol was higher in PBC patients, both NC and HC, than in NC and HC control subjects, though the difference was not significant. Mean 27-hydroxycholesterol concentrations in serum were significantly lower in HC-PBC patients when compared to HC controls who did not respond to the diet ($P < 0.05$). Serum levels of 27-hydroxycholesterol were significantly higher in HC controls than in NC controls ($P < 0.01$).

A good correlation between 27-hydroxycholesterol and cholesterol levels was found for the control subjects ($r = 0.771$, n = 18; $P < 0.001$; **Fig. 1A**), whereas it was much lower in the PBC population ($r = 0.524$, n = 19; $P < 0.05$). Considering the PBC-NC and PBC-HC groups separately, significance of the correlation was maintained in the former ($r = 0.709$, n = 11, $P < 0.01$). In contrast, correlation was not found when only PBC-HC patients were considered ($r = 0.199$, n = 8, Fig. 1B).

DISCUSSION

Our data confirm that cholesterol synthesis and biliary elimination of sterols are impaired in patients with primary biliary cirrhosis and indicate for the first time that the scavenger effect on elevated cholesterol by cholesterol 27-hydroxylase is also impaired in this patient population.

Lathosterol serum levels are considered to be valid indicators of whole-body cholesterol synthesis in humans because they both reflect the activity measured by the sterol balance technique (4) and appear to be related to the activity of hepatic 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase), the key enzyme of cholesterol synthesis (17).

Hypercholesterolemic controls had significantly lower lathosterol serum levels than normocholesterolemic controls, thus indicating a feed-back regulation by elevated cholesterol. As previously reported (3), we found signifi-

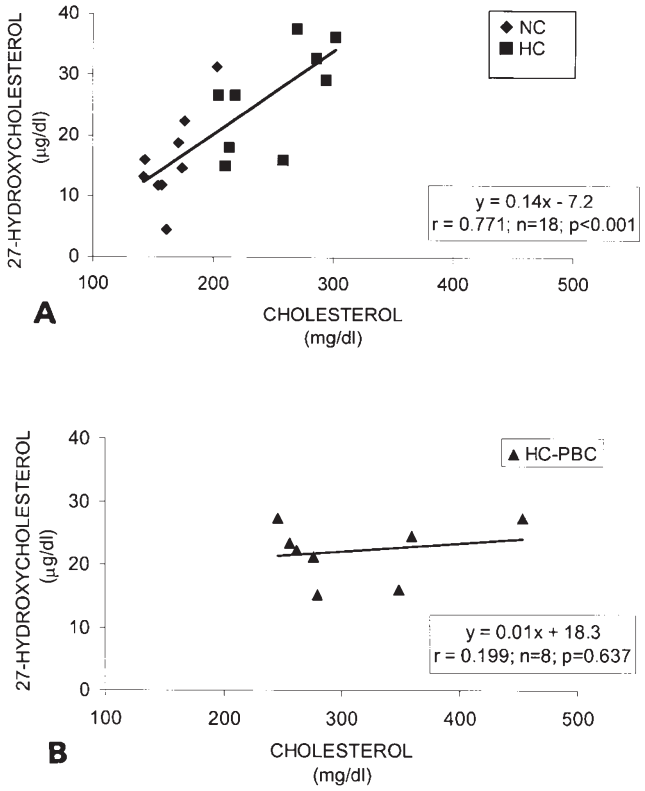

Fig. 1. Correlation between serum 27-hydroxycholesterol and cholesterol levels in control subjects ($P < 0.001$) (A, upper panel) and in HC-PBC patients (B, lower panel).

cantly lower lathosterol serum levels in patients with PBC compared with normocholesterolemic controls and these values were significantly lower in those patients with more severe disease, i.e., serum bilirubin concentrations higher than 2 mg/dl. These observations indicate that an impairment of the liver function rather than a feed-back effect of dietary cholesterol is responsible for reduction of lathosterol serum levels in patients with PBC.

Serum levels of plant sterols are regulated by their intestinal absorption and biliary sterol secretion (5, 18) and have been reported to be positively related with cholesterol absorption while a negative relationship with cholesterol synthesis was found (5). It has been reported by Nikkilä, Hockerstedt, and Miettinen (3) that campesterol and sitosterol serum levels increase significantly in cholestatic patients as compared to controls, whereas serum levels tend to return to normal in patients with end-stage disease. Impaired biliary secretion of sterols may explain the initial increase of serum levels, while lipid malabsorption is responsible for the tendency to normalization at the late stages of the disease. In addition, they found that a decrease of campesterol/sitosterol ratio is related to the disease progression and have suggested that this ratio may be used as an indicator for the need of liver transplantation (3). We found a significant increase in serum levels of campesterol and sitosterol in NC-PBC patients when compared to NC controls, while only sitosterol was significantly higher in the HC-PBC group, $P < 0.01$, than in HC controls (Table 2). Interestingly, campesterol serum levels were significantly higher in patients with increased bilirubin levels compared to the remaining patients, thus indicating that increased serum levels of dietary sterols mainly reflect impairment of their biliary elimination as suggested by Nikkila and Miettinen (19). In both NC and HC patients with PBC, the campesterol to sitosterol ratio (0.40 ± 0.06; $n = 12$ and 0.32 ± 0.04; $n = 8$) was lower than in controls (0.87 ± 0.3; $n = 10$ and 1.6 ± 0.6; $n = 10$) and accounted for 46% and 37% of the respective control groups. These percentages are similar to those previously reported for patients with advanced disease, but this is not the case for our patient population in which only 16% of patients had increased serum bilirubin levels compared to 55% of the population studied by Nikkilä et al. (3). Both absolute (expressed as μg/100 mg total cholesterol) and relative (campesterol to sitosterol ratio) serum concentrations of the two tested dietary sterols differed from those reported for NC controls as mean values were 88 ± 6 for campesterol, 166 ± 29 for sitosterol, and 0.87 ± 0.3 for the campesterol to sitosterol ratio instead of values of 192 ± 28, 125 ± 15, and 1.5 ± 0.1, respectively, which have been reported by Nikkilä, Hockerstedt, and Miettinen (6). Such a discrepancy may be also due to the different dietary habits of the two populations. As a matter of fact, in the Mediterranean diet, fat is mainly assumed as olive oil in which campesterol accounts only for about 4% of sitosterol, whereas in oils other than olive oil it accounts for 12–25%. Therefore, it is rather questionable to assume that the campesterol to sitosterol ratio is a valuable parameter to establish the timing of liver transplantation.

In PBC patients, bile acid synthesis was reported to be significantly reduced compared to healthy subjects. In order to clarify whether this corresponded to a down-regulation of the hepatic activity of the biosynthetic process, we determined serum levels of 7α-hydroxycholesterol which is the product of the rate-limiting step of the process. The increased mean values of this sterol in PBC patients is consistent with an enhancement of cholesterol 7α-hydroxylase activity, as previously observed in bile duct-ligated rats (10, 11). However, the significance level was not reached. In any case, this observation allowed us to conclude that a decrease in bile acid synthesis may not be ascribed to feedback regulation of the enzyme.

In order to understand whether the decreased bile acid synthesis in PBC may depend upon a depression of the extrahepatic formation of bile acids, serum levels of 27-hydroxycholesterol were measured. Mean values found in normocholesterolemic controls were in good agreement with those previously determined by other authors in plasma of healthy volunteers (20). In HC control subjects, 27-hydroxycholesterol concentrations were previously reported to be significantly higher than in NC controls, and to be correlated with serum cholesterol concentration (21), suggesting an up-regulation of cholesterol 27-hydroxylase in circulating cells. Our data also show significantly higher 27-hydroxycholesterol levels in HC controls in respect to NC controls. Nevertheless, a significant correlation was found between serum concentrations of 27-hydroxycholesterol and cholesterol only for control subjects, both NC and HC, but not for PBC patients (Fig. 1A, B). This result is particularly relevant for the interpretation of modifications of cholesterol synthesis and absorption in PBC which appears from data presented here and previously reported by Nikkilä et al. (3, 6, 19). Despite the fact that the contribution of side chain cholesterol oxidation in peripheral tissues to the overall production of bile acids is still undefined, a deficient "scavenger effect" on cholesterol by its hydroxylation to 27-hydroxycholesterol in patients with PBC may induce a down-regulation of cholesterol synthesis and a slower sterol excretion, thus contributing, at least in part, to the low serum levels of lathosterol and the high levels of both campesterol and sitosterol found in these subjects. However, the significant relationship that we have found between the presence of hyperbilirubinemia and serum levels of both lathosterol and dietary sterols suggests that impaired liver function plays a major role.

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