Lipoprotein abnormalities in cholestasis. I.
Electrophoretic and ultracentrifugal analyses.

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Abstract

The alterations of lipid composition in sera of patients with liver diseases, particularly intrahepatic cholestasis and biliary obstruction, were studied by ultracentrifugation and polyacrylamide-gel disc-electrophoresis of lipoproteins and apoproteins. The elevation of serum cholesterol in intrahepatic cholestasis was greater than in biliary obstruction. The appearance of lipoprotein X in obstructive disease accounted for most of the increased cholesterol. The level of non-lipoprotein X cholesterol in intrahepatic cholestasis was significantly elevated, this being in part ascribed to the appearance of a new class of cholestatic lipoprotein, Slow-migrating HDL. The electrophoretic pattern of lipoprotein in cholestasis was generally characterized by a decrease in alpha band intensity and, in some types of cholestasis, by the appearance of Slow-migrating HDL. In addition, other abnormal lipoproteins exhibiting the characteristics of triglyceride-rich LDL (LP-Y), LP-X-like HDL and LDL-like HDL were found in some cases of intrahepatic cholestasis and biliary obstruction.

KEYWORDS: intrahepatic cholestasis, primary biliary cirrhosis, polyacrylamide-gel disc-electrophoresis, high density lipoprotein, Slow-migrating HDL (HDL-S)

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LIPOPROTEIN ABNORMALITIES IN CHOLESTASIS

II. ISOLATION, CHARACTERIZATION AND CLINICAL EVALUATION OF AN ADDITIONAL CHOLESTATIC LIPOPROTEIN (SLOW-MIGRATING HDL)

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Abstract. An additional cholestatic lipoprotein with a slower mobility than the usual α-lipoprotein on polyacrylamide-gel disc-electrophoresis was found in the serum of patients with cholestasis. This abnormal lipoprotein was referred to as Slow-migrating HDL (HDL-S), because it was mostly recovered in the high density lipoprotein (HDL) fraction after preparative ultracentrifugation. HDL-S was precipitated by dextran sulfate and Mg⁺⁺ but did not react with either concanavalin A or anti-β-lipoprotein serum. The main apoprotein of HDL-S was Apo A-I, and a trace of Apo E was also present. HDL-S was relatively enriched in free cholesterol and triglycerides and had a density in the range of 1.063 to 1.083. The appearance of HDL-S in serum or plasma was closely associated with chronic mild intrahepatic cholestasis, particularly as in primary biliary cirrhosis and related conditions.

Key words: intrahepatic cholestasis, primary biliary cirrhosis, polyacrylamide-gel disc-electrophoresis, high density lipoprotein, Slow-migrating HDL (HDL-S)

Several abnormal lipoproteins have been reported in cholestasis; well-known ones include lipoprotein X (LP-X), which was characterized by Seidel et al. (1, 2) and evaluated clinically by Magnani and Alaupovic (3), and triglyceride-rich LDL, initially described as LDL III by Klör et al. (4) and later fully studied by Müller et al. (5) and characterized by Kostner’s group as lipoprotein Y (LP-Y) (6). Although these lipoproteins are found in the low density lipoprotein (LDL) fraction, their existence seems to depend on different pathological mechanisms (4). High density lipoproteins (HDL) in cholestasis are commonly decreased, although the presence of a few cholestatic HDL’s, LP-X-like HDL (7, 8) and LDL-like HDL (9), has also been reported.

An additional cholestatic lipoprotein in HDL fraction, Slow-migrating HDL (HDL-S), was found in our laboratory during the course of analyzing cholestatic lipoproteins by polyacrylamide-gel disc-electrophoresis (PAGE) (10). The present paper deals with the physico-chemical properties and clinical evaluation of...
the new cholestatic lipoprotein of HDL class.

MATERIALS AND METHODS

Blood samples were taken after 12–14 h fasting from 25 cases of intrahepatic cholestasis (viral 5, drug-induced 1, drug-induced, suspected 11, primary biliary cirrhosis (PBC) 4, PBC, suspected 1, anicteric cholestasis with biliary enzyme elevation (PBC-related) 1 and cause unknown 2), 17 cases of malignant, complete biliary obstruction, 44 cases of parenchymal liver diseases (acute viral hepatitis 9, chronic viral hepatitis 4, post-hepatic liver cirrhosis 12, primary hepatoma 10, alcoholic fatty liver 2 and alcoholic liver cirrhosis 7) and 1 case of Caroli's syndrome. All the samples used for characterization of HDL-S were those obtained from a case of PBC. Diagnoses were made by clinical and laboratory findings and further confirmed on most of them by peritoneoscopy and histological examination and/or percutaneous transhepatic cholangiography or endoscopic retrograde cholangiography. EDTA (disodium salt, Sigma Chemical Co., 1 mg/ml blood) was used as an anti-coagulant in blood collection when preparative ultracentrifugation was subsequently performed, otherwise serum was separated, stored at 4°C, and used for assay within one week.

PAGE of lipoproteins was performed according to the method of Naito et al. (11) and that of apoproteins was done by the methods of Kane (12) and of Weber and Osborn (13).

Lipoprotein fractions, very low density lipoprotein (VLDL, 0.95<d<1.006), low density lipoprotein (LDL, 1.006<d<1.063) and high density lipoprotein (HDL, 1.063<d<1.21), were separated by preparative ultracentrifugation at 4–6°C according to the method of Yasugi and Homma (14) using a Hitachi 65P ultracentrifuge.

Hydroxyapatite (Bio-Gel HTP, Bio-Rad Lab.) column chromatography of the HDL fraction was carried out with a linear gradient (0.05–0.55 M) of sodium phosphate buffer with a gradient mixer (GM-1, Pharmacia Fine Chem.) and a column of 9×15 mm in size (K 9/15, Pharmacia Fine Chem.) after dialyzing the fraction overnight against a buffer consisting of 100 mM NaCl, 10 mM Tris and 1 mM EDTA, pH 7.4. Samples were collected by monitoring the absorption at 280 nm with a duo Monitor, Model 1222 (Laboratory Data Control Inc.), dialyzed in a similar manner and concentrated by a concentrator (Minicon-B 15, Amicon Corp.).

A combination of dextran sulfate (Dextran Sulphate, sodium salt, MW 500,000, Pharmacia Fine Chem.) and Mg²⁺ as a bivalent cation was adopted for lipoprotein separation by polyanion precipitation method (15).

Concanavalin A (Con A, No. C-2010, Sigma Chemical Co.) precipitation of lipoproteins was also employed at a final concentration of 30 mg/ml based on the known affinity of Con A to lipoproteins (16).

For immunochemical analyses, anti-α₁ lipoprotein (Apo A-I and A-II), anti-β lipoprotein (Apo B) and anti-LP-X (Apo C) sera were obtained from Behring Inst. and anti-Apo E serum was kindly donated by Dr. G. Utermann, Marburg, West Germany. Immunoreaction was performed in 1% agar (Bacto-Agar, Difco Lab.)
prepared in barbital buffer for 24 h at room temperature.

Lipoproteins were negatively stained and observed under electron microscopy by a routine laboratory technique.

Concentrations of total cholesterol and cholesterol esters were determined by the method of Zak (17), triglycerides by Van Handel and Zilversmidt (18), phospholipids by Bartlett (19) and protein by Lowry et al. (20).

RESULTS

The electrophoretic patterns of lipoproteins obtained from a patient with PBC and separated by preparative ultracentrifugation are presented in Fig. 1.

Fig. 1. PAGE of lipoprotein fractions and original plasma. The columns stand from left to right for plasma, VLDL, LDL, HDL, HDL without HDL-S, and HDL-S (polyanion treatment). HDL-S was precipitated by polyanion treatment and redissolved in 0.1 M sodium citrate.

Fig. 2. PAGE of partially purified HDL-S. A, partially purified HDL-S (1.063<d<1.083); B, whole HDL fraction (1.063<d<1.21); and C, HDL (1.083<d<1.21).

In addition to the usual major lipoprotein bands with pre β, β, and α-mobilities present in corresponding lipoprotein fractions (VLDL, LDL and HDL), a broad lipoprotein band migrating slower than the usual α-lipoprotein band, or just behind the hemoglobin band, was found in the original plasma and predomi-
nantly in the HDL fraction. Although a faint band of similar mobility was also present in the LDL fraction, the unusual lipoprotein was termed as Slow-migrating HDL (HDL-S) because of its major distribution in HDL. HDL-S in HDL fraction was found to be precipitated by dextran sulfate and Mg$^{++}$; however the precipitated HDL-S redissolved in 0.1 M sodium citrate completely lost its mobility, as is shown in the same figure. HDL-S was separated from the normally migrating HDL by re-ultracentrifugation of the HDL fraction at 1.063<d<1.083 and its PAGE pattern was compared with that of the original HDL fraction and of separated HDL (Fig. 2). Although the lipoprotein with $\beta$-pre $\beta$-mobility remained in the HDL fraction after re-ultracentrifugation, it could be removed by subsequent HTP column chromatography due to its high affinity to the HTP.

The HDL from a healthy individual and the partially purified HDL-S from the plasma of PBC appeared in the first peak (0.05–0.15 M) and a trace of the partially purified HDL-S in the second peak (0.20–0.35 M) (Figs. 3A and C).

![Fig. 3. HTP column chromatography of HDL's. A, HDL from a healthy individual; B, from a patient with PBC; and C, partially purified HDL-S. The phosphate gradient is shown by the line.](http://escholarship.lib.okayama-u.ac.jp/amo/vol33/iss5/1)
normal HDL. The HDL-S eluted in the first peak from the partially purified HDL-S was shown to give a single band with original HDL-S mobility on PAGE (result not shown).

HDL-S was precipitated with dextran sulfate and Mg\(^{++}\) but did not react with either Con A or anti-β-lipoprotein serum, as is shown in Fig. 4. This clearly differentiated HDL-S from LDL and HDL (note that the lipoproteins lighter than LDL were all removed by this treatment).

The electrophoretic profiles of apoproteins separated from PBC by using urea are presented in Fig. 5. A single band migrating to the site of Apo A-I to E was found for HDL-S. The band was resolved on sodium dodecyl sulfate (SDS) electrophoresis into two fractions of Apo A-I and E mobilities (Fig. 6). The apoprotein composition of HDL-S was apparently different from that of HDL; i.e., the former had Apo A-I as a major component with a trace of Apo E, while the latter had, in addition, Apo C-I, II and III (Figs. 5 and 6). Accordingly,
the apoprotein composition of HDL in this case was characterized by a relative increase in Apo E compared with the normal apoprotein composition of HDL. The apoprotein content of VLDL was markedly reduced and the LDL had unusual Apo A-I, E and C-III components.

Electron microscopy of the HDL fraction containing HDL-S revealed three types of particles differing in size, as shown in Fig. 7A. After removal of HDL by re-ultracentrifugation and larger particles by HTP chromatography, electron microscopy showed relatively uniform particles of 150–200 Å in diameter (Fig. 7B). Thus, these particles were slightly larger than those of HDL (approximately 100 Å) (Fig. 7C), and comparable in size to those of HDL obtained from healthy person. There were no disc-shaped lipoproteins with lamellar structure apparent in the HDL-S.

For further characterization of HDL-S, immunochemical analyses were made and the results are given in Fig. 8. When each lipoprotein fraction was reacted against anti-Apo E serum, precipitin lines formed with plasma, VLDL, LDL and HDL but not with HDL-S. The lack of a reaction to HDL-S was probably due to the low concentration of HDL-S applied (Figs. 8A and C). HDL-S was precipitated, however, with anti-Apo A serum, confirming the results obtained on PAGE that Apo A was the main apoprotein of HDL-S. Although bile juice from PBC reacted with anti-Apo B, C and E sera and also with the patient's own plasma, it formed no precipitin line with lipoprotein fractions containing LDL from type III hyperlipoproteinemia (21). The specificity of these precipitin lines as immunoprecipitation was not studied further.
Slow-migrating HDL in Cholestasis

The lipid composition of HDL-S prepared by precipitation with dextran sulfate and Mg$^{2+}$ is presented in Table 1. The chemical composition of HDL-S was strikingly different from the HDL's of both patients and controls. The lipid composition of HDL-S was commonly characterized by low contents of phospholipids and esterified cholesterol and a high content of triglycerides as compared with that of HDL's from healthy controls and patients. The free cholesterol content in HDL-S was higher than that in the HDL of patients with positive HDL-S which was also increased as compared with that in HDL of healthy controls. There were no lipoprotein classes with similar lipid and protein compositions in LDL and VLDL of the cases studied.

The incidence of HDL-S positive cases in intrahepatic cholestasis was 14/25 (viral 5/5, drug-induced 1/1, drug-induced, suspected 4/11, PBC 2/4, PBC, suspected 1/1, anicteric cholestasis with biliary enzyme elevation (PBC-related).
Fig. 8. Immunological analyses of lipoprotein fractions. A, lipoprotein fractions, including HDL-S, prepared from the plasma of a patient with PBC were reacted against anti-Apo E serum placed in the center well; B, HDL-S, VLDL, LDL and HDL from PBC reacted with anti-Apo A, B and C sera; C, lipoprotein fractions from a case with type III hyperlipoproteinemia, B bile and plasma from PBC reacted against anti-Apo E serum; D, bile from PBC and VLDL, LDL and HDL from type III hyperlipoproteinemia reacted against anti-Apo A, B and C sera.

**Table 1. Lipid and Protein Composition of HDL-S and Other Lipoproteins**

<table>
<thead>
<tr>
<th>Lipoprotein class</th>
<th>Chemical composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC</td>
</tr>
<tr>
<td>Patients</td>
<td></td>
</tr>
<tr>
<td>HDL-S</td>
<td>7.3</td>
</tr>
<tr>
<td>HDL</td>
<td>12.9</td>
</tr>
<tr>
<td>LDL</td>
<td>18.6</td>
</tr>
<tr>
<td>VLDL</td>
<td>9.9</td>
</tr>
<tr>
<td>Healthy controls</td>
<td></td>
</tr>
<tr>
<td>HDL</td>
<td>17.9</td>
</tr>
<tr>
<td>LDL</td>
<td>25.9</td>
</tr>
<tr>
<td>VLDL</td>
<td>10.0</td>
</tr>
</tbody>
</table>

*a whole HDL fraction including HDL-S. EC, esterified cholesterol; FC, free cholesterol; TG, triglycerides; and PL, phospholipids. Mean values of 3 cases for Patients and 2 cases for Healthy controls. Patients include 1 PBC and 2 intrahepatic cholestasis.

1/1 and cause unknown 0/2), in biliary obstruction 4/17, in other parenchymal liver diseases 0/44 and in Caroli’s syndrome 1/1 (cf. Ref. 22). Moderate to intense bands of HDL-S on PAGE were found for intrahepatic cholestasis and faint bands for biliary obstruction.

Fig. 9. Clinical courses of three cases with intrahepatic cholestasis. A, probably drug-induced intrahepatic cholestasis; B, PBC; and C, chronic hepatitis (acute exacerbation with cholestasis).
The appearance and disappearance of HDL-S in relation to the clinical course were analyzed in three cases of intrahepatic cholestasis (drug-induced, suspected 1, PBC 1 and chronic hepatitis of acute exacerbation with cholestasis 1) (Fig. 9). In a case of intrahepatic cholestasis probably induced by drug (Fig. 9A), the decrease in serum bilirubin roughly paralleled the decrease in LP-X level, followed by decreases in Al-Pase, γ-GTP and LAP in that order. HDL-S became positive shortly after normalization of LP-X while biliary enzymes remained elevated (8 to 13 hospital weeks). During the course of a case of PBC, fluctuating serum bilirubin, LP-X at low levels, and markedly increased Al-Pase, LAP and γ-GTP activities (Fig. 9B), HDL-S was observed while the LP-X or bilirubin levels were kept at a minimum (1–3 and 26–31 weeks). Similarly, in a case of chronic hepatitis superimposed with intrahepatic cholestasis (Fig. 9C), the decreases in serum bilirubin and LP-X levels were followed by the appearance of HDL-S during the period of paradoxical rises in GOT, GPT, LAP and γ-GTP activities, which reflected some sort of hepatic injury (9–27 weeks). HDL-S was found consistently in a case of anicteric cholestasis with biliary

Fig. 10. Distribution of data in several liver function tests and lipid levels among HDL-S positive and negative groups. Mean values are shown by horizontal bars. Each dot represents a single determination for individuals for the HDL-S-positive or negative period during the course of illness.
enzyme elevation, a PBC related entity. Thus, it was apparent that an inverse relationship between LP-X (or bilirubin) and HDL-S levels existed and that HDL-S usually appeared with minimal LP-X level while biliary enzyme levels were still high.

The results of liver function tests and lipid levels in HDL-S positive and negative cases are given in Fig. 10 and Table 2. The results revealed signifi-

<table>
<thead>
<tr>
<th>Cases</th>
<th>Bil* (mg/dl)</th>
<th>ALP (GRU)</th>
<th>LAP (mU/ml)</th>
<th>γ-GTP (Ku)</th>
<th>GOT (μPH)</th>
<th>Ch-Ease (mg/dl)</th>
<th>TC (mg/dl)</th>
<th>TG (mg/dl)</th>
<th>PL (mg/dl)</th>
<th>LP-X* (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDL-S</td>
<td>3.0 ± 17.8</td>
<td>740 ± 163</td>
<td>197 ± 178</td>
<td>0.64 ± 0.18</td>
<td>295 ± 137</td>
<td>37 ± 77</td>
<td>77 ± 17.9</td>
<td>11.2 ± 13.9</td>
<td>504 ± 229</td>
<td>160 ± 64</td>
</tr>
<tr>
<td>(+)</td>
<td>2.7 ± 12.7</td>
<td>350 ± 163</td>
<td>197 ± 178</td>
<td>0.64 ± 0.18</td>
<td>295 ± 137</td>
<td>37 ± 77</td>
<td>77 ± 17.9</td>
<td>11.2 ± 13.9</td>
<td>504 ± 229</td>
<td>160 ± 64</td>
</tr>
<tr>
<td>[−]</td>
<td>10.1 ± 10.8</td>
<td>237 ± 129</td>
<td>0.21</td>
<td>143 ± 86</td>
<td>228 ± 272.4</td>
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* p < 0.001  ** p < 0.01  Values are expressed in mean ± S. D.

dicantly lower serum bilirubin and LP-X levels in HDL-S positive cases as compared with negative ones. Although the differences in serum enzyme levels between these two groups were not statistically significant, the enzyme activities tended to be slightly higher in cases with HDL-S than in those without, this being compatible with the results analyzed in individual cases during the period of clinical follow-up of liver function tests and lipid levels (cf. Fig. 9).

DISCUSSION

The well-known abnormal lipoproteins in cholestatic liver diseases are those found in the LDL class; i.e. LP-X (1-3) and LP-Y (4-6). They appear in the serum of cholestatic patient by different mechanisms. LP-X is formed by regurgitation of bile lipoprotein (23) or in relation to lecithin: cholesterol acyltransferase (LCAT) deficiency (24), while LP-Y appears to result from the decreased hepatic triglyceride lipase activity secondary to the liver damage (5). Higher levels of LP-X are more commonly found in obstructive jaundice and lower levels in intrahepatic cholestasis (3).

On the other hand, little is known of the HDL abnormalities in cholestasis. Although Eder et al. (25), Havel et al. (26) and Furman et al. (27) reported the decrease of HDL level in serum or plasma of various liver diseases in 1950's, the qualitative abnormality of HDL was first demonstrated by Blomhoff (28), who isolated two kinds of HDL from cholestatic patients by Sephadex G-200 gel filtration. Danielsson (29) reported the presence of an abnormal HDL in cholestatic plasma. It was isolated by zonal ultracentrifugation and termed HDL₀ because of its relatively low density among HDL's. Arnesjö et al. (30) charac-
terized the HDL in human cholestasis after separating HDL by gel filtration and designated it HDL₁-C.

Electron microscopic abnormalities of HDL in alcoholic hepatitis, in which cholestasis tends to occur, was demonstrated by Sabesin et al. (31). Further physico-chemical characterization as a nascent HDL was made recently by the same group (32).

The abnormal cholestatic HDL reported in this paper was discovered (10) on PAGE because of its slower mobility as compared with HDL of healthy person. Since it was mostly recovered in the HDL fraction by ultracentrifugation, it was termed Slow-migrating HDL (HDL-S). Besides of its slow migration on PAGE, it differed from normal HDL in possessing higher contents of triglycerides, high ratios of free cholesterol to esterified cholesterol, apoprotein composition of predominant Apo A-I with a trace of Apo E, the reactivity with dextran sulfate to form a precipitate, and had larger particles as seen by electron microscopy. Although these distinct properties were found in HDL-S, similarities to normal HDL also existed; that is the spherical shape under electron microscopy and the co-elution with HDL on HTP column chromatography. Thus, the relatively low content of Apo E and the spherical shape of HDL-S are markers to differentiate it from a nascent HDL.

Hamilton et al. (24) reported that the nascent HDL accumulating in rat liver perfusates in the presence of LCAT inhibitor contained decreased levels of cholesteryl esters and protein, and was enriched in unesterified cholesterol and phospholipids. Electron microscopy of a negatively stained preparation of the nascent HDL revealed disc-shaped lipid bilayer particles with frequent rouleaux formation. The major apoprotein was arginine-rich protein (or Apo E) with relatively small amounts of Apo A-I. The nascent HDL in human plasma from a patient with alcoholic hepatitis has also been shown by Ragland et al. (32) to have unusually large amounts of arginine-rich protein and a deficiency or near absence of Apo A-I. The relative proportion of arginine-rich protein and Apo A-I in the nascent HDL of subjects with alcoholic hepatitis varied depending on the stage of the disease. Several abnormal lipoproteins such as LP-X-like lipoprotein have been reported by Glomset et al. (33) in patients with familial LCAT deficiency. Havel et al. (34) and Norum et al. (35) have obtained evidence suggesting that arginine-rich protein is transferred from nascent HDL to VLDL by LCAT reaction during the course of lipoprotein metabolism. It appears, therefore, that the proportion of Apo E to Apo A-I changes at various stages of the maturation process of HDL depending on the degree of the decrease in LCAT activity caused by liver damage. Thus, it is expected that no marked reduction of LCAT activity is present in cases with positive HDL-S. In fact, LCAT activity in the serum of a HDL-S positive case was within normal limits (un-
published observation), a finding compatible with the apoprotein composition of HDL-S; i.e. a major Apo A-I and a minor Apo E. In this sense, HDL-S can be regarded as “dysmatured” HDL. The appearance of HDL-S in some cholestatic patients could be explained by assuming the production of abnormal Apo A, as is suggested by Seidel et al. (36). However, the abnormality of Apo A which they observed is the impaired capacity of Apo A to bind neutral lipids. The fraction containing Apo A does not stain for lipids, thus differing somewhat from what the present study revealed.

Another mechanism that HDL-S might be formed from regurgitated bile lipoprotein lacks evidence, although a precipitin line was found between anti-Apo E serum and bile without further characterization.

The lipid composition of HDL-S is similar to that of nascent HDL (32) except for the presence of relatively abundant triglycerides. Increased concentration of free cholesterol in cholestasis in spite of normal LCAT activity has been reported by Blomhoff (28), although normal concentration of cholesteryl esters was found in his case. At present, no suitable explanation is available for the high triglycerides and unesterified cholesterol contents in the presence of LCAT activity in HDL-S positive patients. If Apo E transfer not mediated by LCAT reaction exists, HDL-S might be produced from the disturbance of Apo E transfer from nascent HDL to VLDL, thus receiving more triglycerides by some mechanism.

Close similarities exist between HDL₁-C described by Arnesjö et al. (30) and HDL-S characterized in the present study; both had lower density and slower mobility compared with normal HDL, major apoproteins of A-I, E and C-I and clinical conditions of prolonged cholestasis for the appearance of the new class of HDL. However, the amount of Apo E was much less in HDL-S than in HDL₁-C and the clinical picture of mild cholestasis in addition to longstanding cholestasis characterizes better the clinical course of HDL-S positive cases. The maximum level of HDL-S in our cases was 70 mg/dl, which is comparable to that of HDL₁-C.

The clinical significance of HDL-S lies in the fact that it appears in mild cholestasis of unusual cases, which are characterized by minimum bilirubin elevation with relatively high levels of biliary enzymes and transaminases, and by low levels of LP-X. It is, therefore, unlikely that HDL-S is formed by regurgitation of bile lipoprotein into the plasma. It rather represents a facet of inductive phase of intrahepatic cholestasis, as revealed by high levels of biliary enzymes; thus, this enables the formation and excretion of abortive or immature HDL as HDL-S.

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Slow-migrating HDL in Cholestasis