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Esterification of 4β-hydroxycholesterol and other oxysterols in human plasma occurs independently of LCAT

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Abstract **The acyltransferase LCAT mediates FA esterification of plasma cholesterol. In vitro studies have shown that LCAT also FA-esterifies several oxysterols, but in vivo evidence is lacking. Here, we measured both free and FA-esterified forms of sterols in 206 healthy volunteers and 8 individuals with genetic LCAT deficiency, including familial LCAT deficiency (FLD) and fish-eye disease (FED). In the healthy volunteers, the mean values of the ester-to-total molar ratios of the following sterols varied: 4β-hydroxycholesterol (4βHC), 0.38; 5,6α-epoxycholesterol (5,6αEC), 0.46; 5,6β-epoxycholesterol (5,6βEC), 0.51; cholesterol, 0.70; cholestane-3β,5α,6β-triol (CT), 0.70; 7-ketocholesterol (7KC), 0.75; 24S-hydroxycholesterol (24SHC), 0.80; 25-hydroxycholesterol (25HC), 0.81; 27-hydroxycholesterol (27HC), 0.86; and 7α-hydroxycholesterol (7αHC), 0.89. In the individuals with LCAT deficiency, the plasma levels of the FA-esterified forms of cholesterol, 5,6αEC, 5,6βEC, CT, 7αHC, 7KC, 24SHC, 25HC, and 27HC, were significantly lower than those in the healthy volunteers. The individuals with FLD had significantly lower FA-esterified forms of 7αHC, 24SHC, and 27HC than those with FED. It is of note that, even in the three FLD individuals with negligible plasma cholesteryl ester, substantial amounts of the FA-esterified forms of 4βHC, 5,6αEC, 7αHC, 7KC, and 27HC were present. We conclude that LCAT has a major role in the FA esterification of many plasma oxysterols but contributes little to the FA esterification of 4βHC. Substantial FA esterification of 4βHC, 5,6αEC, 7αHC, 7KC, and 27HC is independent of LCAT.**

Supplementary key words cholesterol/acyltransferase • inborn error of metabolism • lipoprotein metabolism • oxidized lipids • enzyme • liquid chromatography • tandem mass spectrometry • high density lipoprotein/metabolism • sterols • chronic kidney disease • lecithin:cholesterol acyltransferase

Three forms of cholesterol are present in the plasma: free cholesterol (FC), FA cholesteryl ester (CE), and cholesterol sulfate. CE is generated by esterification of cholesterol by LCAT, which catalyzes the transfer of the PC acyl group at the *sn*-2 position to the hydroxyl moiety at the C3 position of cholesterol (1). Cholesterol in discoidal HDL, also called nascent HDL or preβ1-HDL, is preferentially esterified by LCAT (2). This subfraction of HDL is largely composed of apoAI after apoAI accepts cholesterol via ABCA1 from cells such as hepatocytes and macrophages (3). Excess cholesterol in the peripheral tissues is converted to CE by LCAT. Therefore, LCAT is considered to be critical for reverse cholesterol transport, which transfers excess cholesterol in the peripheral tissues to the liver for elimination into bile (4). Cholesterol sulfate, which is generated by sulfonation of cholesterol by cholesterol sulfotransferase (SULT2B1b) (5), is a minor component comprising about 0.1% of total cholesterol (TC) (6).

It has been shown that LCAT catalyzes the transfer of the acyl group not only to cholesterol but also to pregnenolone, dehydroepiandrosterone (7), and various oxysterols (8). Oxysterols are oxygenated 27-carbon molecules derived from cholesterol enzymatically (9) or nonenzymatically and can be potent biologically active molecules with diverse functions (10–12). Thus far, the 3β-hydroxyl group of several oxysterols has been shown to accept the acyl moiety to form monoesters in vitro (8, 13, 14). These oxysterols include 5,6α-epoxycholesterol (5,6αEC), 5,6β-epoxycholesterol (5,6βEC), cholestane-3β,5α,6β-triol (CT), 7α-hydroxycholesterol (7αHC), 7β-hydroxycholesterol (7βHC), 7-ketocholesterol (7KC), 24S-hydroxycholesterol (24SHC), 25-hydroxycholesterol (25HC), and 27-hydroxycholesterol (27HC). The 27-hydroxy group of 27HC can be esterified to form diester (8). Indeed, 80% of 24SHC and 84% of 27HC were present in esterified forms in the plasma of healthy volunteers (15).

LCAT deficiency syndromes are rare diseases arising either from mutations of LCAT or from neutralizing antibodies against LCAT proteins (16). They are characterized by a severe decrease of plasma HDL (hypo-α-lipoproteinemia), corneal opacity, anemia, and renal dysfunctions including proteinuria and impaired glomerular filtration. To date, over 100 mutations have been reported as pathogenic variants in

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the LCAT gene (17). Some mutations cause severe full-blown clinical manifestations, such as familial LCAT deficiency (FLD), while other mutations cause a milder phenotype called fish-eye disease (FED) that is restricted to corneal opacity during the life span. In FLD, the mutant LCAT enzyme is either absent in plasma or nonfunctional. Previously, it was proposed that FED is caused by a functionally abnormal LCAT that esterifies cholesterol on lipoproteins containing apoB (β-activity) but not on HDL (α-activity) (18). FED was proposed to be caused by a mutant LCAT with milder functional impairment (19). Previous studies showed that large molecular weight LDL (20) or lipoprotein-X (21, 22) present in the LCAT-deficient patients contributed to the renal phenotype in FLD. We recently reported that two lipoprotein fractions (Lp8 and Lp12-16), identified by HPLC with a gel filtration column, are specific to the renal phenotype (23). In addition, Karuna et al. (24) reported that patients heterozygous for an LCAT mutation were found to have lower concentrations of total 27HC in HDL compared with unaffected family members. However, it remains unclear whether LCAT deficiency affects the FA esterification of various oxysterols.

In order to determine how much of each oxysterol is FA esterified in the plasma and whether LCAT is involved in its production, we measured the plasma levels of both free and FA-esterified forms of oxysterols in the plasma of 206 healthy volunteers as well as in eight LCAT-deficient patients and correlated the values with the clinical phenotype of LCAT deficiency.

For this study, we measured nine oxysterols: 4β-hydroxycholesterol (4βHC), 5,6αEC, 5,6βEC, CT, 7αHC, 7KC, 24SHC, 25HC, and 27HC. All of the oxysterols can be ligands for nuclear receptors such as LXRs, retinoic acid-related orphan receptors (RORs), estrogen receptor, glucocorticoid receptor, and arylhydrocarbon receptor (25, 26). Most of 5,6αEC and 5,6βEC are produced nonenzymatically, while 5,6αEC is produced enzymatically (27). While CT, 7KC, 7αHC, and 25HC are produced by both enzymatic and nonenzymatic pathways, 4βHC, 24SCH, and 27HC are produced exclusively by enzymatic pathways (10, 12). 5,6αEC is a precursor of dendrogenin A, a tumor suppressant (28). Both 5,6αEC and 5,6βEC are metabolized to CT, which is a precursor of 6-oxocholestan-3β,5α-diol, a tumor promotor in breast cancer (29); 24SHC is related to neurodegenerative diseases (30); 27HC is related to atherosclerosis (31) or breast cancer (32, 33); 25HC can modulate viral infection (34, 35), osteoarthritis (36) and ER stress in macrophages deficient in neutral cholesterol ester hydrolase 1 (NCEH1) (37), which is also known as KIAA1363 or arylacetamide deacetylase-like 1 (AADACL1). 4βHC can be used as a marker of cytochrome P450 3A activity (38). Their sources and putative functions are summarized in supplemental Table S1.

MATERIALS AND METHODS

Sample collection

Blood was collected from healthy human volunteers (Table 1) and LCAT-deficient patients (Table 3) (23) after they had fasted for 10 h. The clinical characteristics of the patients have been reported previously (39–44). These patients did not take any medications that significantly induce cytochrome P450 3A4 expression, such as carbamazepine or phenobarbital (38). After centrifugation at 1,500 *g* for 15 min, serum samples were stored at −80°C until analyses. TC and FC were measured by Determiner L TC II kit and Determiner L FC kit (Kyowa Medex, Co., Ltd.), respectively. LCAT activity was measured by Anasolv LCAT kit (Sekisui Medical Co., Ltd.). The experimental protocol was approved by the ethics committees of Jichi Medical University, Utsunomiya Higashi Hospital, and Chiba University Graduate School of Medicine. Informed consent was obtained from all subjects, and the experimental procedures were conducted in accordance with the ethical standards of the Helsinki Declaration.

Sample preparation

Serum oxysterols including 4βHC, 5,6αEC, 5,6βEC, CT, 7αHC, 7KC, 24SHC, 25HC, and 27HC were quantified by LC-MS/MS essentially as described by Honda et al. (45). Briefly, deuterated oxysterols, including [$^2H_7$]4βHC (5 ng), [$^2H_7$]5,6αEC (5 ng), [$^2H_7$]5,6βEC (5 ng), [$^2H_7$]CT (1 ng), [$^2H_7$]7αHC (5 ng), [$^2H_7$]KC (5 ng), [$^2H_6$]24SHC (2.5 ng), [$^2H_3$]25HC (1 ng), [$^2H_7$]27HC (5 ng), and 5 μg of dibutylhydroxytoluene were added to 20 μl of serum as internal standards. Portions of the mixtures were saponified in 0.5 ml of 1 N ethanolic KOH with butylated hydroxytoluene at 37°C for 1 h for obtaining the values for total sterols. After the addition of 0.25 ml of distilled water, sterols were extracted with 1 ml of n-hexane, and the extract was evaporated to dryness under nitrogen gas. The sterols were derivatized to the picolinyl esters as described previously (24). The reagent mixture for derivatization consisted of 2-methyl-6-nitrobenzoic anhydride (100 mg), 4-dimethylamino-pyridine (30 mg), picolinic acid (80 mg), pyridine (1.5 ml), and triethylamine (200 μl). Freshly prepared reagent mixture (170 μl) was added to the sterol extract, and the reaction mixture was incubated at 80°C for 60 min. After the addition of 1 ml of n-hexane, the mixtures were centrifuged at 1,500 *g* for 5 min. The clear supernatant was collected and evaporated at 80°C under nitrogen gas. The residue was dissolved in 50 μl of acetonitrile, and an aliquot (5 μl) was injected into the LC-MS/MS system (described below).

LC-MS/MS analysis

The LC-MS/MS system consisted of a TSQ Quantum Ultra mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with an H-ESI probe and an Ultimate 3000 HPLC system (Thermo Fisher Scientific). The samples were separated on a Hypersil GOLD column (150 × 2.1 mm, 3 μm; Thermo Fisher Scientific) at 40°C at a flow rate of 300 μl/min. The mobile phase was programmed to linearly change from acetonitrile-methanol-water (40:40:20, v/v/v) containing 0.1% acetic acid to acetonitrile-methanol-water (45:45:10, v/v/v) containing 0.1% acetic acid over 20 min. The final mobile phase was kept constant for an additional 20 min. The general LC-MS/MS conditions were as follows: spray voltage, 1,000 V; vaporizer temperature, 350°C; sheath gas (nitrogen) pressure, 85 psi; auxiliary gas (nitrogen) flow, 60 arbitrary units; ion transfer capillary temperature, 350°C; collision gas (argon) pressure, 1.5 m Torr; and ion polarity, positive. Oxysterols were quantified by selected reaction monitoring using the characteristic precursor-product ion transition under optimized collision energy, as previously described (45). We performed assays using 10 different concentrations of standard oxysterols varying from 200 pg/ml to 2 μg/ml. The coefficient variance was less than 0.20 at 1 ng/ml and more of CT and 25HC; 2 ng/ml or more of 27HC; 5 ng/ml or more of 4βHC, 5,6αEC, 5,6βEC, 7KC, and 24SHC; and 10 ng/ml or more of 7αHC, validating the sensitivity of our assays (supplemental Table S2).

The amounts of the sterols that were esterified with FA were calculated by subtracting the values for nonsaponified samples from the values for saponified samples. These values are referred to as esterified forms and used to calculate ester-to-total molar ratios. To estimate the net weight of the esterified sterols, the above values were multiplied by 1.68.

Statistics

All data are presented as mean ± SD. GraphPad Prism software was used for data analyses. Unpaired Mann-Whitney test, Pearson correlation test, and ANOVA with Bonferroni multiple-comparison test were used for comparisons as appropriate. Differences were considered significant for P values <0.05.

RESULTS

Oxysterols in healthy subjects

Table 1 summarizes the clinical characteristics of the healthy volunteers. More men were recruited than women. The average age was 47 years and the average BMI was 22.7 kg/m^2. Sex differences were observed for BMI, waist circumference, systolic and diastolic blood pressure, fasting plasma glucose, HDL-C, non-HDL-C, and TG.

Plasma levels of total, free, and FA-esterified forms of cholesterol and oxysterols and their ester-to-total molar ratios are shown in **Table 2**. As for total values, plasma 4βHC levels were higher in women than in men as reported previously (38, 46), while plasma levels of 7KC and 27HC were higher in men than in women. A similar sex difference was previously reported for 27HC (47–49). As for ester-to-total molar ratios, the values for men and women for 7KC (0.76 vs. 0.75) and 27HC (0.86 vs. 0.85) were nearly the same and were combined for the following analyses.

The relative ratios of ester-to-total molar ratios for cholesterol and oxysterols are compared in Table 2 and **Fig. 1**. The ester-to-total molar ratios for cholesterol (CE/TC ratio), CT, 7αHC, 7KC, 24SHC, 25HC, and 27HC were distributed in a relatively narrow range with mean values of 0.70, 0.70, 0.89, 0.75, 0.80, 0.81, and 0.86, respectively. These values were mutually significantly different except between 4βHC and 5,6αEC, between 4βHC and 5,6βEC, between cholesterol and CT, between 7αHC and 27HC, and between 24SHC and 25HC. By contrast, the ester-to-total molar ratios for 4βHC, 5,6αEC, and 5,6βEC were distributed over a wider range from 0.01 to 0.91, from 0.04 to 0.88, and from 0.08 to 0.82 with the mean value of 0.38, 0.46, and 0.51, respectively, which was significantly lower than the values for the other oxysterols and cholesterol.

Comparison of cholesterol and oxysterols between the healthy subjects and LCAT-deficient patients

Supplemental Table S3 shows plasma levels of total, free, ester, and ester-to-total molar ratios of cholesterol and the oxysterols in individual patients. The plasma levels of total values for cholesterol, 5,6αEC, 5,6βEC, CT, 7αHC, 7KC, 24SHC, 25HC, and 27HC, but not 4βHC, were significantly lower in the LCAT-deficient patients (five with FLD and three with FED) than in the healthy volunteers (**Fig. 2**). The plasma levels of total 7αHC, 24SHC, and 27HC were significantly lower in patients with FLD than in patients with FED.

The plasma levels of the free forms of 7KC, 24SHC, and 25HC were significantly higher in the LCAT-deficient patients than in the healthy volunteers, while those of 5,6αEC and 5,6βEC were significantly lower in the LCAT-deficient patients than in the healthy volunteers (**Fig. 3**). There were no significant differences in those of 4βHC, CT, 7αHC, and 27HC. However, most of the values for 7KC, 24SHC, and 25HC overlapped between the LCAT-deficient patients and the healthy volunteers. None of the free forms of any of the oxysterols had plasma levels that were significantly different between FLD and FED patients.

The plasma levels of the FA-esterified forms of cholesterol, 5,6αEC, 5,6βEC, CT, 7αHC, 7KC, 24SHC, 25HC, or 27HC, but not of 4βHC, in the LCAT-deficient patients were significantly lower than those in the healthy volunteers (**Fig. 4**). The values for 5,6βEC, CT, 7αHC, or 25HC did not overlap between the LCAT-deficient patients and the healthy volunteers. The plasma levels of the FA-esterified forms of 7αHC, 24SHC, and 27HC in the patients with FLD were significantly lower than those in the patients with FED. The values for cholesterol, 24SHC, or 27HC did not overlap between the patients with FLD and the healthy volunteers.

The free-to-total molar ratios of cholesterol, 4βHC, 5,6αEC, 5,6βEC, CT, 7αHC, 7KC, 24SHC, 25HC, and 27HC were significantly higher in the LCAT-deficient patients than in the healthy volunteers (**Fig. 5**). However, the values

TABLE 1. Clinical characteristics of the healthy volunteers

	All	Males	Females	P
Age (years)	47.0 ± 9.2	46.6 ± 9.3	47.9 ± 9.1	0.231
BMI (kg/m^2)	22.7 ± 3.1	23.1 ± 2.8	21.9 ± 3.3	0.003
Waist circumference (cm)	80.1 ± 8.2	81.8 ± 7.8	77.8 ± 8.5	0.015
Systolic blood pressure (mmHg)	113.1 ± 16.0	116.1 ± 16.1	107.8 ± 14.3	<0.0001
Diastolic blood pressure (mmHg)	70.0 ± 11.7	73.2 ± 11.5	64.4 ± 9.9	<0.0001
Fasting glucose (mg/dl)	92.6 ± 7.0	94.2 ± 6.9	89.6 ± 6.2	<0.0001
HbA1c (%)	5.6 ± 0.3	5.6 ± 0.2	5.6 ± 0.3	0.970
TC (mg/dl)	188.1 ± 29.8	188.2 ± 31.0	188.0 ± 27.5	0.565
HDL-C (mg/dl)	64.3 ± 16.4	59.6 ± 14.1	72.9 ± 16.7	<0.0001
Non-HDL-C (mg/dl)	135.1 ± 41.1	142.0 ± 35.0	131.3 ± 29.1	0.0212
LDL-C (mg/dl)	124.7 ± 30.7	127.4 ± 32.1	119.9 ± 27.2	0.0572
TG (mg/dl)	110.1 ± 73.8	119.4 ± 78.7	93.4 ± 61.8	<0.0001
LCAT activity (nmol/ml/h)	183.1 ± 121.2	185.8 ± 122.2	178.2 ± 119.2	0.6671

The clinical values were compared among males (n = 132) and females (n = 74). Data are presented as mean ± SD.

TABLE 2. Plasma levels of total, free, and FA-esterified oxysterols, and ester-to-total molar ratios in healthy volunteers

	All	Males	Females	*P*
Cholesterol				
Total	188.1 ± 29.8	188.2 ± 31.0	188.0 ± 27.5	0.9076
Free	57.1 ± 12.3	57.2 ± 12.8	56.8 ± 11.4	0.8235
Ester	131.0 ± 23.4	131.0 ± 25.0	131.2 ± 20.2	0.6264
Ratio	0.70 ± 0.06	0.69 ± 0.07	0.70 ± 0.04	0.7684
4βHC				
Total	21.9 ± 12.2	20.1 ± 8.9	25.1 ± 15.9	0.0278
Free	13.0 ± 6.3	12.0 ± 5.8	14.7 ± 6.9	0.0083
Ester	9.0 ± 8.5	8.1 ± 6.0	10.5 ± 11.6	0.4319
Ratio	0.38 ± 0.18	0.39 ± 0.19	0.4 ± 0.16	0.4564
5,6αEC				
Total	31.8 ± 17.4	32.2 ± 18.6	31.2 ± 14.9	0.8000
Free	15.8 ± 7.6	15.9 ± 8.1	15.6 ± 6.5	0.6551
Ester	16.0 ± 12.4	16.3 ± 12.9	15.5 ± 11.4	0.9375
Ratio	0.46 ± 0.15	0.46 ± 0.22	0.47 ± 0.19	0.9317
5,6βEC				
Total	179.7 ± 58.2	176.6 ± 59.5	185.2 ± 55.4	0.3322
Free	87.1 ± 37.3	88.8 ± 40.9	84.0 ± 29.7	0.7757
Ester	92.6 ± 44.2	87.8 ± 40.7	101.2 ± 48.7	0.1575
Ratio	0.51 ± 0.17	0.50 ± 0.20	0.53 ± 0.10	0.1741
CT				
Total	102.8 ± 26.6	100.7 ± 26.8	106.5 ± 25.8	0.1511
Free	30.9 ± 10.1	30.2 ± 9.9	32.2 ± 10.3	0.1466
Ester	71.9 ± 20.7	70.5 ± 20.9	74.3 ± 20.0	0.3352
Ratio	0.70 ± 0.16	0.70 ± 0.13	0.70 ± 0.10	0.9908
7αHC				
Total	153.4 ± 181.3	170.6 ± 166.3	122.6 ± 201.7	0.983
Free	13.2 ± 8.9	13.8 ± 9.6	12.1 ± 7.4	0.3803
Ester	140.1 ± 177.1	156.8 ± 161.6	110.5 ± 198.5	0.8085
Ratio	0.89 ± 0.06	0.90 ± 0.06	0.87 ± 0.07	0.0821
7KC				
Total	180.6 ± 65.2	181.5 ± 68.9	179.0 ± 58.0	<0.0001
Free	43.0 ± 14.9	42.2 ± 14.5	44.6 ± 15.4	0.3957
Ester	137.6 ± 54.2	139.3 ± 58.1	134.5 ± 46.2	<0.0001
Ratio	0.75 ± 0.06	0.76 ± 0.06	0.75 ± 0.05	0.0002
24SHC				
Total	49.1 ± 12.2	48.0 ± 12.1	51.2 ± 12.3	0.0561
Free	9.7 ± 3.2	9.3 ± 3.3	10.4 ± 3.0	0.0143
Ester	39.4 ± 11.4	38.7 ± 11.5	40.8 ± 11.0	0.1789
Ratio	0.80 ± 0.07	0.80 ± 0.08	0.79 ± 0.06	0.1036
25HC				
Total	12.7 ± 5.5	13.0 ± 5.8	12.2 ± 4.9	0.2039
Free	2.2 ± 0.9	2.3 ± 0.9	2.2 ± 0.8	0.7988
Ester	10.5 ± 5.2	10.7 ± 5.5	10.0 ± 4.5	0.2110
Ratio	0.81 ± 0.07	0.81 ± 0.07	0.81 ± 0.06	0.7121
27HC				
Total	123.5 ± 33.1	133.3 ± 32.2	106.1 ± 27.0	<0.0001
Free	16.7 ± 4.2	17.6 ± 4.4	15.1 ± 3.4	<0.0001
Ester	106.8 ± 30.8	115.6 ± 30.0	91.0 ± 25.4	<0.0001
Ratio	0.86 ± 0.03	0.86 ± 0.03	0.85 ± 0.04	0.0198

Plasma levels of total, free, and esterified oxysterols, and ratios were compared between males (n = 132) and females (n = 74). Data are presented as mean ± SD.

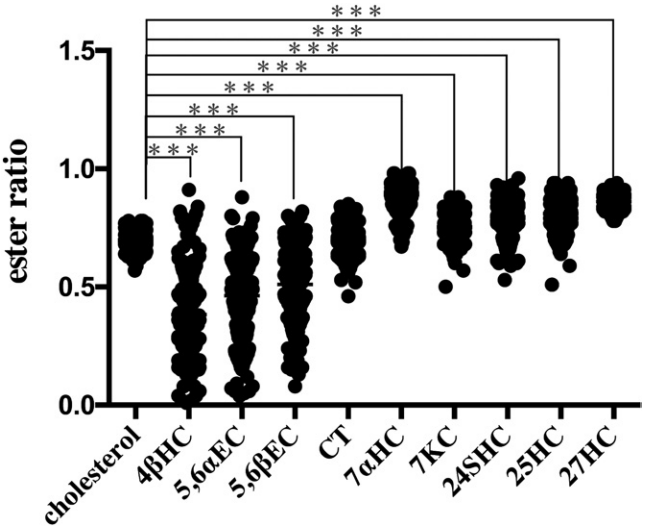

Fig. 1. Comparison of ester-to-total molar ratios of cholesterol and oxysterols in the healthy volunteers. The data were compared between each ester-to-total molar ratio by using repeated-measures ANOVA with Bonferroni multiple comparison test. Plasma ester-to-total molar ratios of healthy volunteers were compared, and these values were mutually significantly different at $P < 0.001$ except between 4βHC and 5,6αEC, between 4βHC and 5,6βEC, between cholesterol and CT, between 7αHC and 27HC, and between 24SHC and 25HC. Data are presented as mean ± SD. ***$P < 0.001$.

for 4βHC, 5,6αEC, or 5,6βEC in the LCAT-deficient patients overlapped with those in the healthy volunteers. The free-to-total molar ratios of cholesterol, 24SHC, or 27HC in the FLD patients were significantly higher than those in the FED patients. The values for cholesterol, CT, 24SHC, 25HC, or 27HC in the FLD patients did not overlap with those in the healthy volunteers. These results suggest that the high plasma levels of the free forms of cholesterol, 24SHC, or 27HC contribute to the renal phenotype of FLD.

We obtained the values for ester either by simply subtracting the values for free from the values for total (method 1) or by further multiplying these values by 1.68 (method 2). The ester-to-total ratios according to method 1 are molar ratios of sterol moieties, while the ester-to-total ratios according to the method 2 are ratios of esterified sterol (weight)/[free sterol (weight) + esterified sterol (weight)]. We present the results of ester-to-total molar ratios in **Fig. 6**. These are essentially identical to the mirror images of Fig. 5. We also present the results of the ester-to-total weight ratios in supplemental Fig. S1. Figure 5 is almost indistinguishable from supplemental Fig. S1. It is of note that the levels of statistical significance of the differences were the same.

We further compared oxysterols between FLD patients with negligible CE/TC molar ratios (complete LCAT deficiency, open squares in Fig. 6) and those with the CE/TC molar ratios more than 0.1 (partial LCAT deficiency, closed squares in Fig. 6). The FLD patients with complete LCAT deficiency significantly differed from the FLD patients with partial LCAT deficiency only in the free 24SHC (Fig. 3) and the 24SHC free-to-total or ester-to-total molar ratio (Fig. 6).

Correlation of CE/TC molar ratio with ester-to-total molar ratio of oxysterols in the LCAT-deficient patients

The finding that the FA ester-to-total molar ratios for cholesterol, 24SHC, and 27HC were significantly lower in the FLD patients than in the FED patients raises the possibility that these ratios are related to LCAT activities. The CE/TC molar ratios, which ranged from 0 to 0.57 in the LCAT-deficient patients, should be proportional to the residual LCAT activities. Interestingly, the LCAT activities measured by the in vitro enzymatic assay were virtually undetectable in the LCAT-deficient patients (**Table 3**). Although it cannot be ruled out that the samples lost LCAT activities during storage, it is more likely that the CE/TC

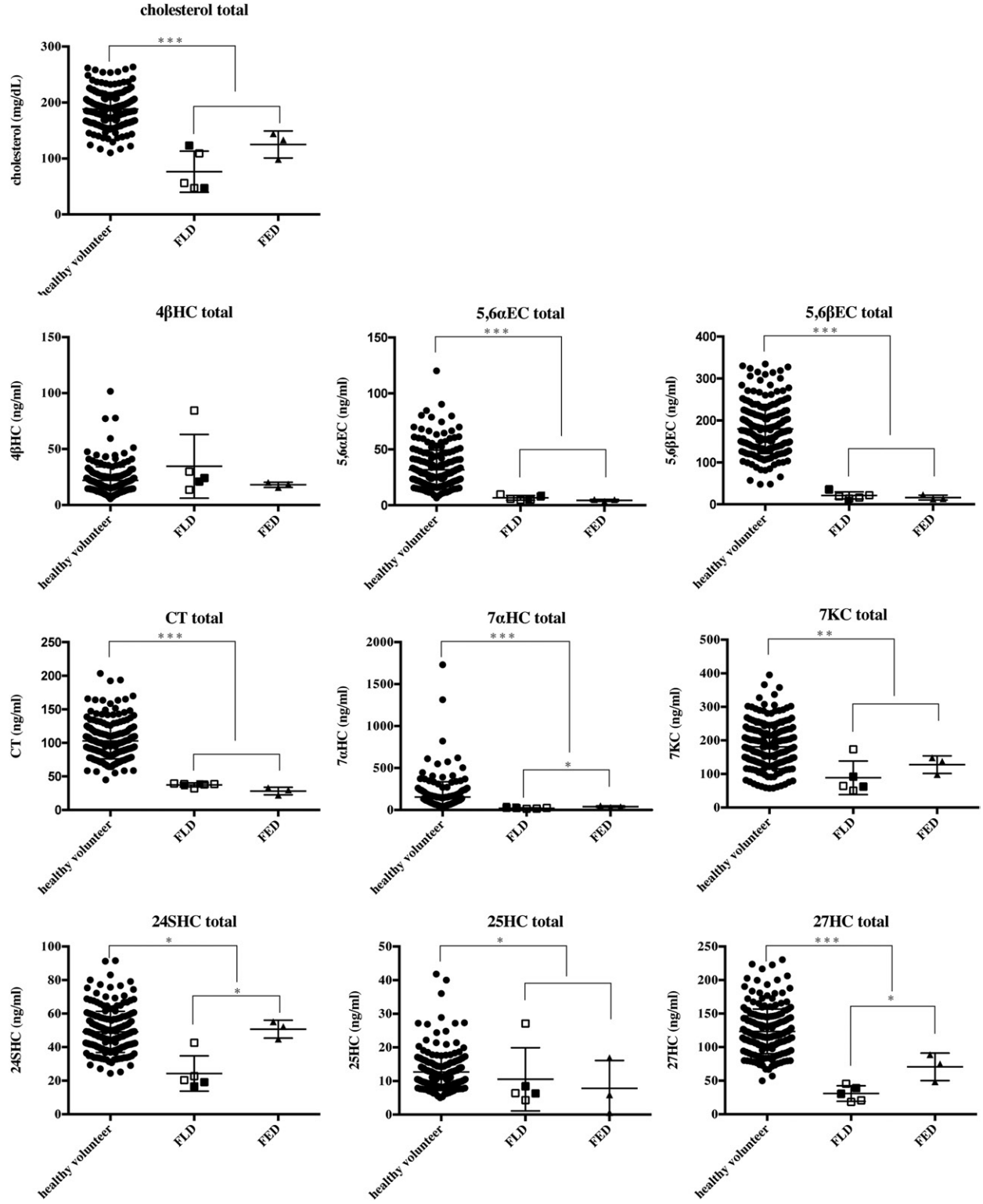

Fig. 2. Plasma levels of total sterols in the healthy volunteers and LCAT-deficient patients. Plasma levels of total sterols were compared among healthy volunteers (closed circles, n = 206) versus FLD + FED (n = 8), complete deficient FLD (open squares, n = 3) and partial deficient FLD (closed squares, n = 2) versus FED (closed triangles, n = 3). Data are presented as mean ± SD. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

molar ratios are much more sensitive than the enzymatic assay. To test this hypothesis, we examined the correlation between the ester-to-total molar ratios of oxysterols and the CE/TC molar ratios. The CE/TC molar ratios were significantly and positively correlated with the ester-to-total molar ratios for 7KC, 24SHC, and 27HC (**Fig. 7**). Among the three oxysterols, 24SHC showed the most striking correlation. By contrast, the CE/TC molar ratios were not significantly

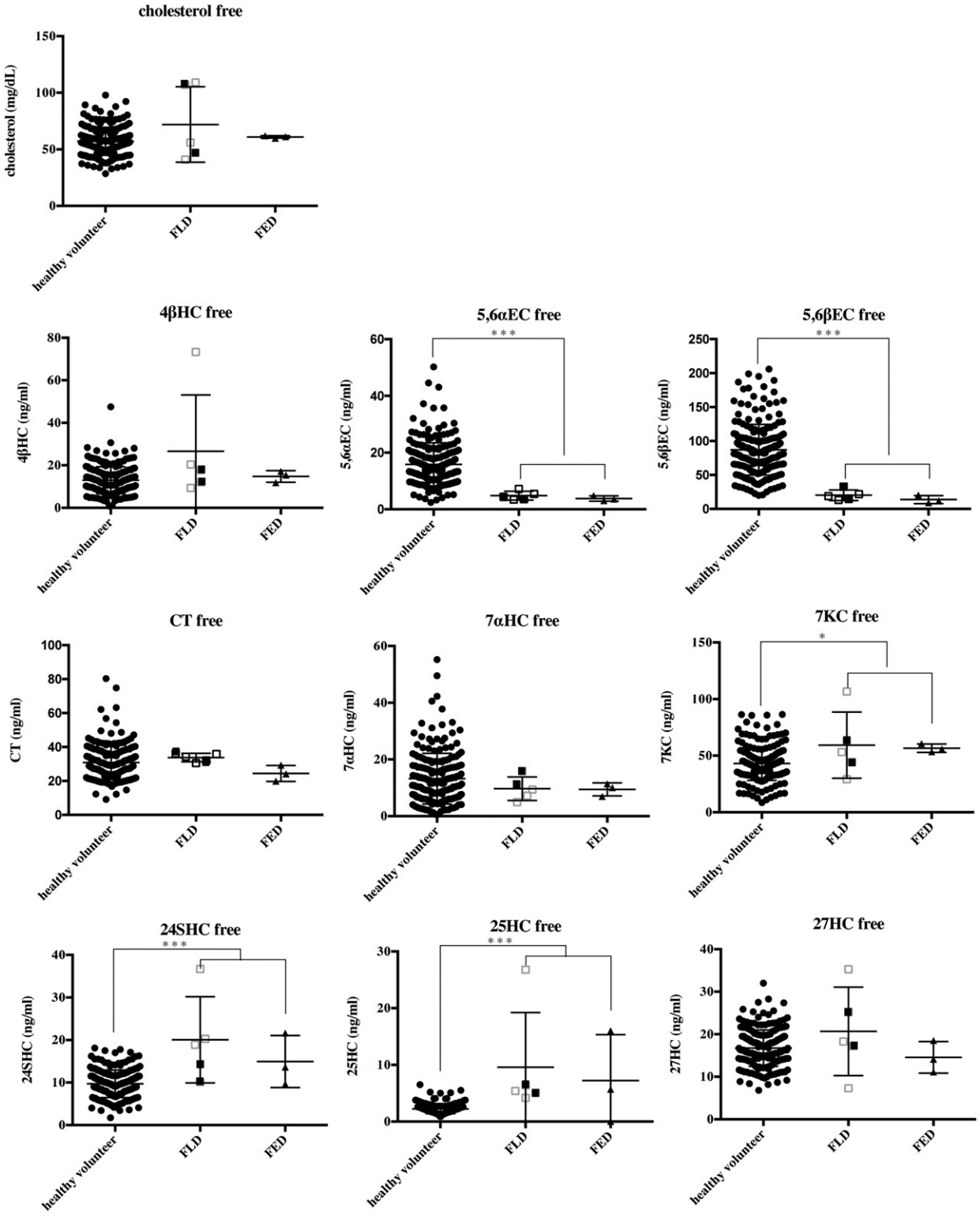

Fig. 3. Plasma levels of free sterols in the healthy volunteers and LCAT-deficient patients. Plasma levels of free sterols were compared among healthy volunteers (closed circles, n = 206) versus FLD + FED (n = 8), complete deficient FLD (open squares, n = 3) and partial deficient FLD (closed squares, n = 2) versus FED (closed triangles, n = 3). Data are presented as mean ± SD. $*P < 0.05$ and $***P < 0.001$.

correlated with the ester-to-total molar ratios for 4βHC, 5,6αEC, 5,6βEC, CT, 7αHC, or 25HC (Fig. 7).

All of the three FLD patients with negligible plasma cholesteryl ester showed the residual ester-to-total molar ratios less than 0.2 only for 5,6βEC, CT, 24SHC, and 25HC. In other words, substantial amounts of the FA-esterified forms of 4βHC, 5,6αEC, 7αHC, 7KC, and 27HC were present in these FLD patients. Based on the relative

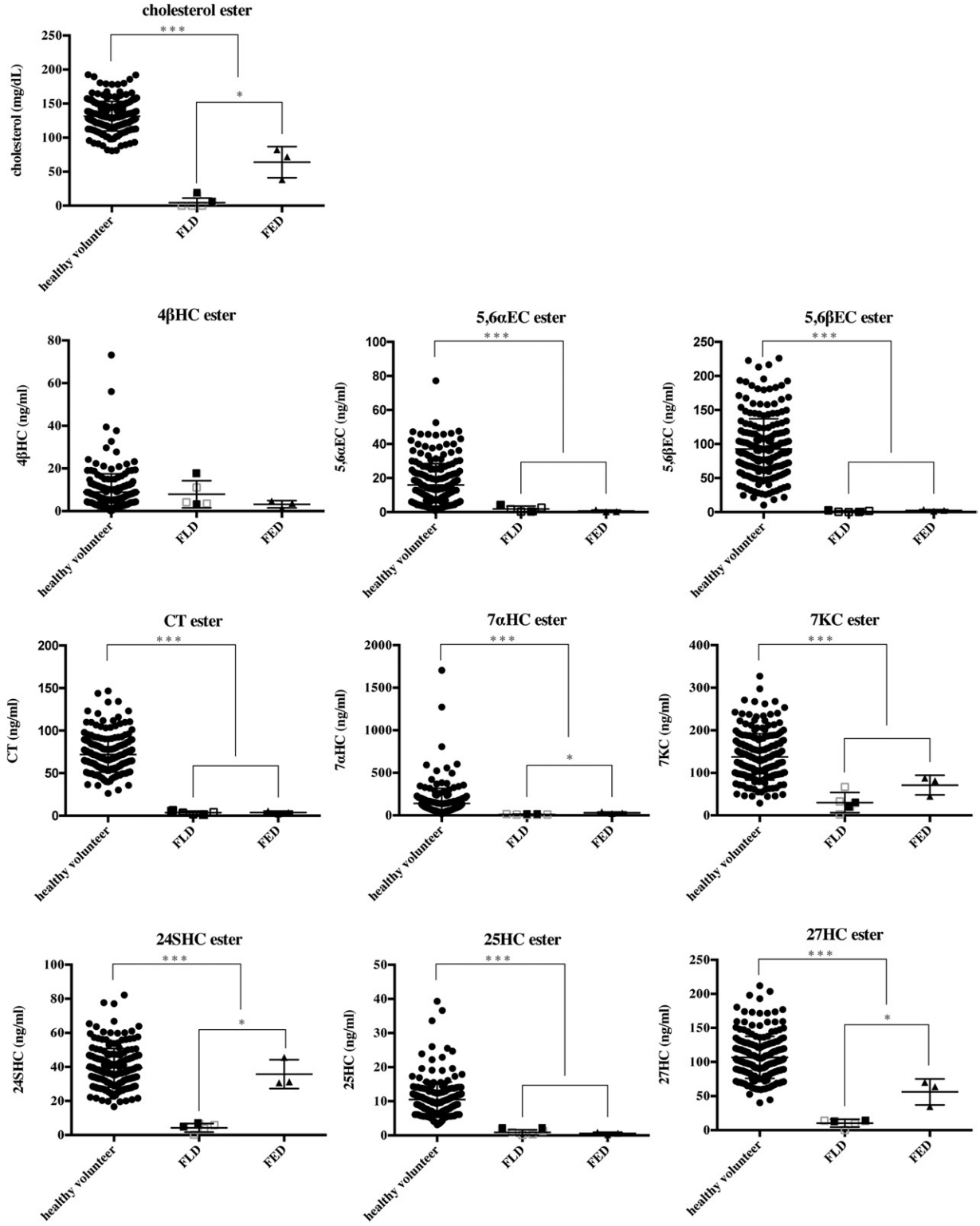

Fig. 4. Plasma levels of FA-esterified sterols in the healthy volunteers and LCAT-deficient patients. Plasma levels of free sterols were compared among healthy volunteers (closed circles, n = 206) versus FLD + FED (n = 8), complete deficient FLD (open squares, n = 3) and partial deficient FLD (closed squares, n = 2) versus FED (closed triangles, n = 3). Data are presented as mean ± SD. $*P < 0.05$ and $***P < 0.001$.

contribution of LCAT-dependent and -independent pathways to their FA esterification (Figs. 6, 7), the sterols can be categorized into the following three groups: *1*) dominantly esterified by LCAT (cholesterol, 5,6βEC, CT, 24SHC, and 25HC); *2*) esterified by both LCAT-dependent and -independent pathways (5,6αEC, 7αHC, 7KC, and 27HC); and *3*) dominantly esterified by LCAT-independent pathway (4βHC).

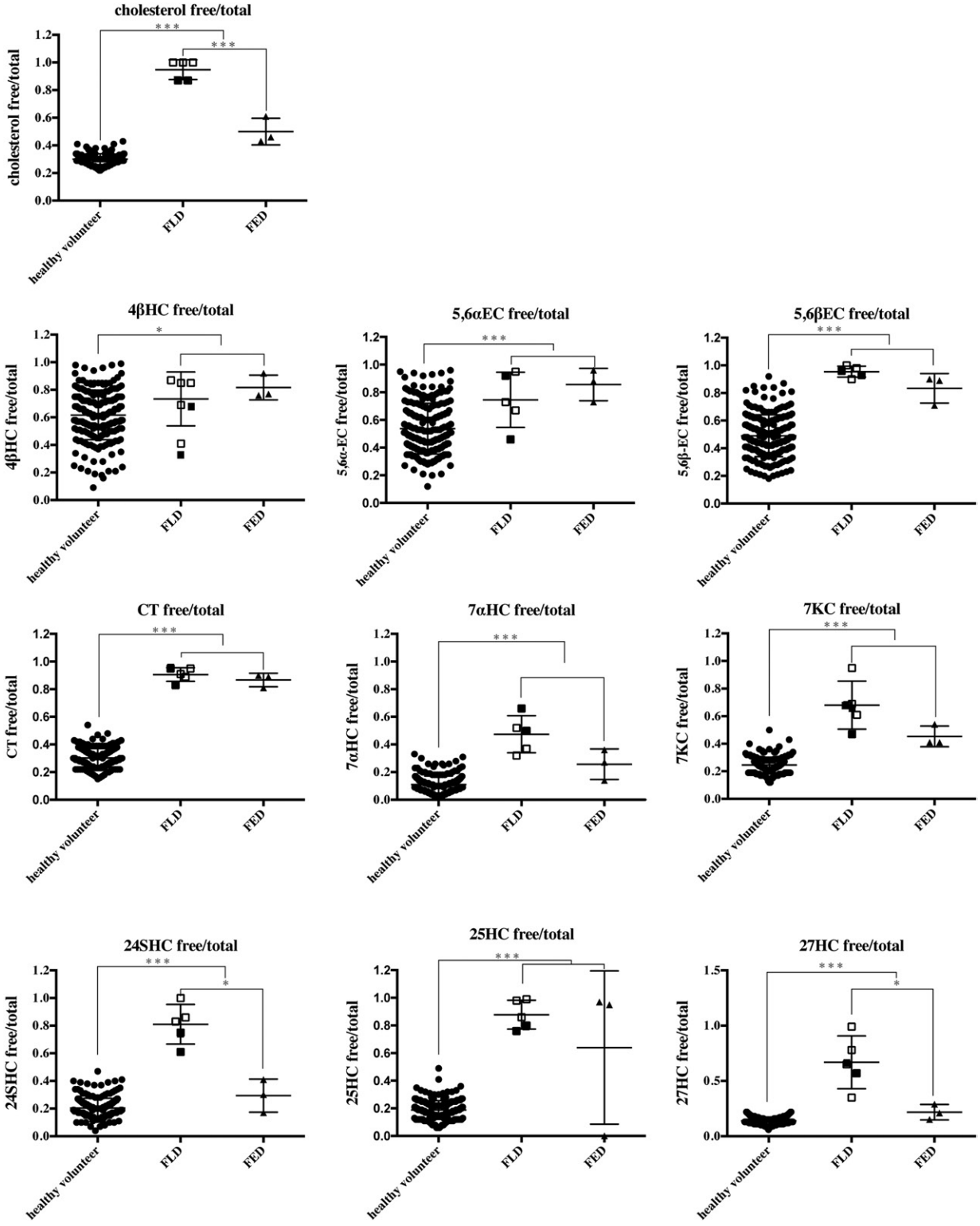

Fig. 5. Free-to-total molar ratios of plasma sterols in the healthy volunteers and LCAT-deficient patients. Free-to-total molar ratios of plasma sterols were compared among healthy volunteers (closed circles, n = 206) versus FLD + FED (n = 8), complete deficient FLD (open squares, n = 3) and partial deficient FLD (closed squares, n = 2) versus FED (closed triangles, n = 3). Data are presented as mean ± SD. $^{*}P < 0.05$ and $^{***}P < 0.001$.

DISCUSSION

In the present study, we show that the ester-to-total molar ratios of plasma CT, 7αHC, 7KC, 24SHC, 25HC, and 27HC were narrowly distributed and close to the plasma CE/TC molar ratio in the healthy volunteers. However, the plasma ester-to-total molar ratios of 4βHC, 5,6αEC, and 5,6βEC were more widely distributed and significantly lower than the plasma CE/TC molar ratio (Fig. 1). The LCAT-deficient patients had higher concentrations of free

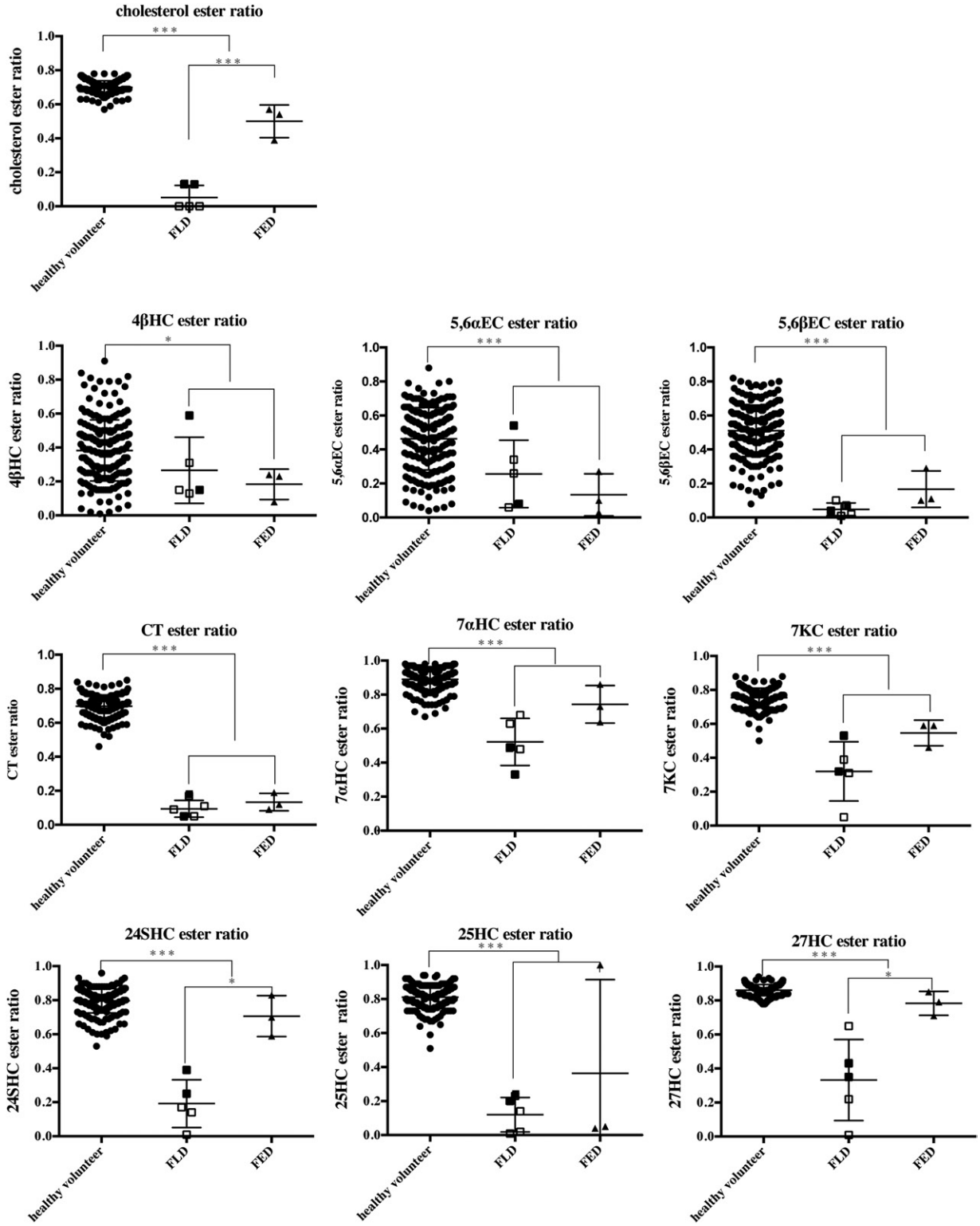

Fig. 6. Ester-to-total molar ratios of plasma sterols in the healthy volunteers and LCAT-deficient patients. Ester-to-total molar ratios of plasma sterols were compared among healthy volunteers (closed circles, n = 206) versus FLD + FED (n = 8), complete deficient FLD (open squares, n = 3) and partial deficient FLD (closed squares, n = 2) versus FED (closed triangles, n = 3). Data are presented as mean ± SD. *P < 0.05 and ***P < 0.001.

7KC, 24SHC, and 25HC than the healthy volunteers (Fig. 3); the LCAT-deficient patients had lower concentrations of FA ester of cholesterol and oxysterols except 4βHC than the healthy volunteers (Fig. 4); the LCAT-deficient patients had lower concentrations of the ester-to-total molar ratios of cholesterol and all oxysterols than the healthy volunteers (Fig. 6). In the LCAT-deficient patients, the patients with FLD had significantly lower ester-to-total molar ratios of 24SHC and 27HC than the patients with FED (Fig. 6). In summary, the present results confirmed in

TABLE 3. Clinical and molecular characteristics of the LCAT-deficient patients

Number	Sex	Age (y)	Ethnicity	Renal Failure, Proteinuria	Hb (g/dl)	Type	TC (mg/dl)	TG (mg/dl)	HDL-C (mg/dl)	CE/TC	LCAT activity (nmol/ml/h)	Reference
1	F	17	Morocco[a]	6 g/24 h	11.4	FLD	109	179	5.8	0	<5	(39)
2	F	61	Japanese	2 g/24 h	9.5	FLD	123	307	9.3	0.13	<5	(40)
3	F	12	Morocco[a]	0.45g/l	9.2	FLD	47	56	10.1	0	<5	(39)
4	F	63	Japanese	0.23 g/24 h	10.3	FLD	47	89	6.3	0.13	<5	(41)
5	M	68	Japanese	0.5g/l	6.6	FLD	56	59	2.0	0	<5	(42)
6	M	58	Dutch[a]	—	—	FED	133	120	4.7	0.54	<5	None
7	M	36	Dutch[a]	—	—	FED	144	205	3.9	0.57	<5	(43)
8	F	30	Dutch[a]	—	—	FED	98	118	4.9	0.39	<5	(44)

This table is reproduced from our previous report (23). All patients had corneal opacity. The mutations were described in (20).

[a]Caucasian.

vitro observations that many of plasma oxysterols are esterified by LCAT, but they also suggest that some of them, 4βHC in particular, are FA esterified by LCAT-independent pathways.

Esterified oxysterols

Previous studies showed that 5,6αEC, 5,6βEC, 7αHC, 7KC, 24SHC, 25HC, or 27HC can be FA esterified by LCAT in vitro (8, 13, 14). The finding that the plasma levels of FA-esterified 5,6αEC, 5,6βEC, 7αHC, 7KC, 24SHC, 25HC, and 27HC were markedly reduced in LCAT-deficient patients clearly demonstrates that LCAT also esterifies oxysterols in vivo (Fig. 4).

Although the ester-to-total molar ratios of these oxysterols were similar to the CE/TC molar ratio, the values were not identical. For example, the ester-to-total molar ratios of 7αHC and 27HC were greater than the CE/TC molar ratio, suggesting that 7αHC and 27HC are more efficiently esterified by LCAT than cholesterol. However, in the initial 15 min, LCAT is reported to FA-esterify cholesterol faster than it esterifies 27HC (8). The yields of monoester products from 27HC were indistinguishable from the yields of CE, regardless of the types of PCs that was used. If 27HC is present in the plasma longer than cholesterol, its FA-esterified form will accumulate more abundantly than CE. However, this is unlikely because the plasma half-life of 27HC (0.75 h) was much shorter than that of cholesterol (65 days) (50, 51), refuting this possibility. Likewise, plasma half-lives of other oxysterols are shorter than that for cholesterol: 0.5 h for 7αHC, 14 h for 24SHC (52), and ∼60 h for 4βHC (53). Therefore, the differences of the ester-to-total molar ratios in oxysterols might not result from the differences in the efficiency of FA esterification by LCAT.

Instead, we hypothesize that most oxysterols are FA esterified by more than one pathway: the LCAT pathway and one or more LCAT-independent pathways. Indeed, the ester-to-total molar ratios of certain oxysterols (4βHC, 5,6αEC, 7αHC, 7KC, or 27HC) were more than 0.2 even in some of the FLD patients with negligible plasma cholesteryl ester, while those of certain oxysterols (5,6βEC, CT, 24SHC, or 25HC) were less than 0.2 in all of the patients (Figs. 6, 7). The ester-to-total molar ratios of 7αHC, 7KC, or 27HC were higher than the CE/TC molar ratio in the healthy volunteers (Fig. 1), probably because these oxysterols are FA esterified not only by LCAT but also by one or more LCAT-independent pathways. The ester-to-total molar ratio of 4βHC was lower than the CE/TC molar ratio, probably because the FA esterification of 4βHC is primarily mediated by an LCAT-independent pathway.

Why didn't deficiency of LCAT affect the ester-to-total molar ratio of 4βHC? Physicochemical experiments showed that 4βHC resembles cholesterol in terms of translocation between biological compartments. Transfer of side chain oxidized species from erythrocytes to plasma occurred at a rate ∼30- to 50-fold faster than cholesterol and the transfer of 7-oxygenated species was found to occur 5-fold faster (54). On the other hand, translocation of 4βHC was even slower than that of cholesterol, thus showing more "cholesterol-like" kinetics. Despite this similarity in the kinetics, 4βHC may not be as good a substrate for LCAT as cholesterol and other oxysterols. This is because the 4β position is very close to the 3β position, and therefore the hydroxyl moiety at the 4β position might interfere with the binding of 4βHC to a putative cholesterol binding site in LCAT via steric hindrance.

How is 4βHC esterified? The esterifying enzyme may also be associated with lipoproteins. A proteome analysis of HDL showed that the major enzymes in HDL are LCAT, paraoxonase-1 (PON1), platelet-activating factor acetyl hydrolase (PAF-AH), also known as lipoprotein-associated phospholipase A2 (LpPLA2), and glutathione selenoperoxidase-3 (GSPx-3) (55). Three enzymes other than LCAT are not known as acyltransferases. Therefore, it is unlikely that 4βHC is FA esterified by an enzyme associated with lipoproteins.

Some oxysterols may also be FA esterified in the liver and directly secreted into the circulation as a component of VLDL or FA esterified in the intestine and secreted as a component of chylomicrons. Indeed, 7αHC, 7KC, 24SHC, 25HC, or 27HC was shown to be esterified by ACAT-1 and ACAT-2 (56). Some oxysterols can also be sulfonated by steroid/sterol sulfotransferase SLUT2B1b (5) to produce oxysterol sulfate.

Free oxysterols

There were no differences in the plasma levels of free forms of cholesterol, 4βHC, CT, 7αHC, or 27HC between the healthy subjects and the LCAT-deficient patients (Fig. 3). On the other hand, the plasma levels of free forms of 7KC, 24SHC, or 25HC were significantly higher in the LCAT-deficient patients than in the healthy subjects. What

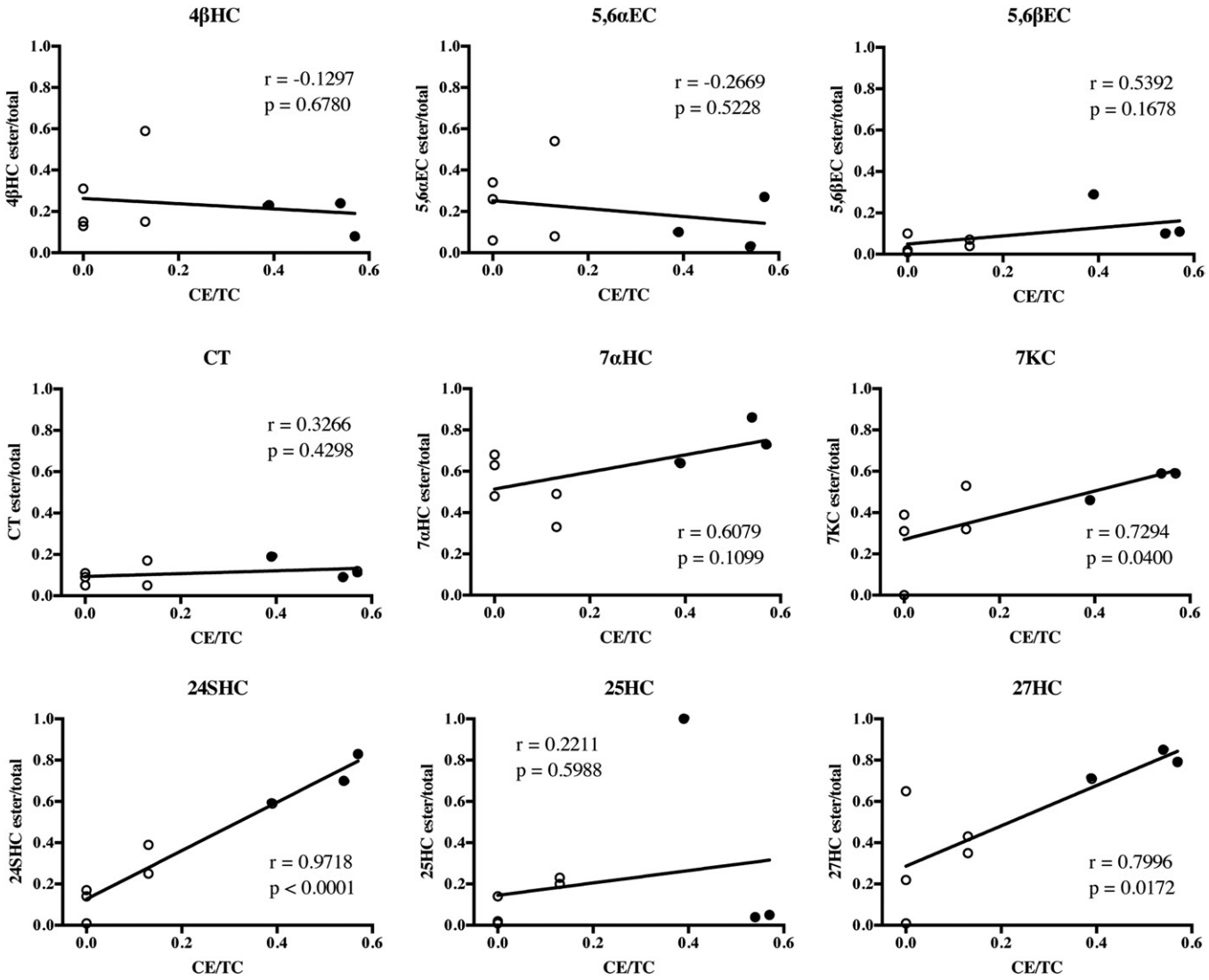

Fig. 7. Correlation between the ester-to-total molar ratios of oxysterols and CE/TC molar ratio in the LCAT-deficient patients. The ester-to-total molar ratios of oxysterols were correlated with the CE/TC molar ratios in the LCAT-deficient patients [FLD (open circles, n = 5) and FED (closed circles, n = 3)]. 7KC, 24SHC, and 27HC were positively associated with CE/TC level.

caused the free forms of 7KC, 24SHC, and 25HC to increase in the LCAT-deficient patients? Because HDL has anti-oxidative properties, the lack of HDL in the LCAT-deficient patients might be expected to stimulate autoxidation, thereby converting cholesterol to certain types of oxysterols, such as 7αHC, 7KC, and 25HC. However, this seems unlikely because the levels of oxidation products of arachidonic acid and linoleic acid and immune-reactive oxidized phospholipids were not significantly different in LCAT-deficient patients (57), presumably refuting the hypothesis that free oxysterols were increased by autoxidation. Indeed, 24SHC is not produced by autoxidation. It is more likely that free forms of these oxysterols accumulate in the plasma because of their defective metabolism due to the deficiency of LCAT, which might be the rate-limiting enzyme in the catabolism of certain oxysterols. Free forms of 7αHC or 27HC will not increase because they are catabolized to bile acids in the liver (9). In this case, LCAT is not rate-limiting.

Oxysterol metabolism and clinical manifestations of LCAT deficiency

Because free oxysterols are known to be cytotoxic (58), increases in plasma levels of free 7KC, 24HC, and 25HC in the LCAT-deficient patients (Fig. 3) are involved in the development of corneal pathologies. However, this possibility may not be high, because the levels of these oxysterols in the LCAT-deficient patients overlapped with those in the healthy volunteers (Fig. 3). We therefore propose that the extremely low plasma levels of certain oxysterols could be responsible for the clinical manifestations of LCAT deficiency, such as corneal opacity and renal dysfunction. In this context, it is noteworthy that certain oxysterols may serve as ligands of nuclear receptors such as LXRs, RORs, estrogen receptor, glucocorticoid receptor, and arylhydrocarbon receptor (supplemental Table S1) (10, 25, 26). If the normal function of cornea or kidney relies on the supply of such ligands from the circulating esterified oxysterols, a deficiency of the esterified forms of oxysterols may

be responsible for the pathologies. A low 25HC FA ester level might be the determinant of corneal opacity in both FLD and FED because the plasma levels of 25HC FA ester were invariably lower in both FLD and FED than in healthy subjects (Fig. 4). In addition, a low 24SHC FA ester level might be the determinant of renal dysfunction, because the plasma levels of 24SHC FA ester were invariably lower in FLD than in FED and healthy subjects (Fig. 4). However, we need to be careful not to overstate the causal relationship.

In conclusion, the FA esterification of 5,6βEC, CT. 24SHC, and 25HC in plasma is dominantly mediated by LCAT as it is for cholesterol, while that of 5,6αEC, 7αHC, 7KC, and 27HC is mediated by both LCAT-dependent and -independent pathways. Changes in the levels of these oxysterols may contribute to the development of some of clinical manifestations of LCAT deficiency. However, the FA esterification of 4βHC is mediated by an LCAT-independent pathway.

Data availability

The datasets generated during and/or analyzed during the current study are available from Daisuke Yamamuro (Jichi Medical University, d.yamamuro@jichi.ac.jp) or Shun Ishibashi (Jichi Medical University, ishibash@jichi.ac.jp) on reasonable request.

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations

CT, cholestane-3β,5α,6β-triol; 5,6αEC, 5,6α-epoxycholesterol; 5,6βEC, 5,6β-epoxycholesterol; FC, free cholesterol; FED, fish-eye disease; FLD, familial LCAT deficiency; 4βHC, 4β-hydroxycholesterol; 7αHC, 7α-hydroxycholesterol; 25HC, 25-hydroxycholesterol; 27HC, 27-hydroxycholesterol; 7KC, 7-ketocholesterol; ROR, retinoic acid-related orphan receptor; 24SHC, 24S-hydroxycholesterol; TC, total cholesterol.

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