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Karuna, R.; Holleboom, A.G.; Motazacker, M.M.; Kuivenhoven, J.A.; Frikke-Schmidt, R.; Tybjaerg-Hansen, A.; ... ; Eckardstein, A. von

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Plasma levels of 27-hydroxycholesterol in humans and mice with monogenic disturbances of high density lipoprotein metabolism

Ratna Karuna[a,b], Adriaan G. Holleboom[c], Mohammad M. Motazacker[c], Jan Albert Kuivenhoven[c], Ruth Frikke-Schmidt[d], Anne Tybjaerg-Hansen[d], Spiros Georgopoulos[e], Miranda van Eck[f], Theo J.C. van Berkel[f], Arnold von Eckardstein[a,b,g,*,1], Katharina M. Rentsch[a,b,g,1]

[a] Institute of Clinical Chemistry, University Hospital Zurich, CH-8091 Zurich, Switzerland
[b] Competence Center of Systems Physiology and Metabolic Diseases, ETH Zurich and University of Zurich, CH-8093 Zurich, Switzerland
[c] Department of Experimental Vascular Medicine, Academic Medical Center, 1105 AZ Amsterdam, The Netherlands
[d] Department of Clinical Biochemistry, Rigshospitalet, Copenhagen University Hospital, 2100 Copenhagen, Denmark
[e] Department of Cell Biology, Biomedical Research Foundation, Academy of Athens, 11527 Athens, Greece
[f] Division of Biopharmaceutics, Leiden/Amsterdam Center for Drug Research, Leiden University, 2333CC Leiden, The Netherlands
[g] Center of Integrated Human Physiology, University of Zurich, CH-8093 Zurich, Switzerland

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ABSTRACT

Secretion of 27-hydroxycholesterol (27OHC) from macrophages is considered as an alternative to HDL-mediated reverse transport of excess cholesterol. We investigated 27OHC-concentrations in plasma of humans and mice with monogenic disorders of HDL metabolism. As compared to family controls mutations in the genes for apolipoprotein A-I, ATP binding cassette transporter (ABC) A1 and lecithin:cholesterol acylstransferase (LCAT) were associated with reduced concentrations of both HDL-cholesterol and HDL-27OHC whereas mutations in the genes for cholesterylester transfer protein (CETP), scavenger receptor type BI and hepatic lipase were associated with elevated HDL concentrations of either sterol. Compared to family controls and relative to the concentrations of total 27OHC and cholesterol, lower 27OHC-ester but normal cholesterylester levels were found in HDL of heterozygous LCAT mutation carriers and nonHDL of heterozygous CETP mutation carriers. In family controls, LCAT activity and CETP mass were more strongly correlated with 27OHC-ester than cholesterylester concentrations in HDL and nonHDL, respectively. These findings suggest that the formation and transfer of 27OHC-esters are more sensitive to reduced activities of LCAT and CETP, respectively, than the formation and transfer of cholesterylesters. 27OHC plasma levels were also decreased in apoA-I-, ABCA1- or LCAT-knockout mice but increased in SR-BI-knockout mice. Transplantation of ABCA1- and/or ABCG1-deficient bone marrow into LDL receptor deficient mice decreased plasma levels of 27OHC. In conclusion, mutations or absence of HDL genes lead to distinct alterations in the quantity, esterification or lipoprotein distribution of 27OHC. These findings argue against the earlier suggestion that 27OHC-metabolism in plasma occurs independently of HDL.

1. Introduction

27-Hydoxycholesterol (27OHC) is a metabolite of cholesterol formed by the mitochondrial cytochrome P450 sterol 27-hydroxylase (CYP27), an enzyme particularly expressed in the vascular endothelium, macrophages and the liver [1,2]. Introduction of a hydroxyl group allows the otherwise hydrophobic cholesterol molecule to pass amphiphilic membranes more easily [2,3]. Because of these physicochemical properties, 27OHC has been postulated to be secreted from cells independently of transporters and extracellular lipoprotein acceptors and thereby to facilitate an alternative route for apolipoprotein (apo) A-I/high density lipoproteins (HDL)-mediated transport of cholesterol from macrophages to the liver [2]. In the liver, 27OHC is an important intermediary product of the so-called alternative bile acid synthesis pathway which contributes ~10% to *de novo* bile acid biosynthesis [2]. In addition, 27OHC is an important ligand of at least two types of nuclear hormone receptors. It activates liver-X-receptors (LXR) alpha and beta which regulate the transcription of several genes involved in lipid and lipoprotein metabolism [4,5]. Most recently 27OHC was identified as the first endogenous selective estrogen receptor modulator

* Corresponding author at: Institute of Clinical Chemistry, University Hospital Zurich, Rämistrasse 100, CH-8091 Zurich, Switzerland. Tel.: +41 44 255 22 60; fax: +41 44 255 45 90.
E-mail address: arnold.voneckardstein@usz.ch (A. von Eckardstein).
[1] Equal contribution to this work.

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(SERM). Both *in vitro* and *in vivo* 27OHC was found to modulate the transcriptional activity of estrogen receptors tissue-specifically either as an agonist or antagonist [6].

27OHC is the most abundant oxysterol in the circulation [4], while plasma levels of 27OHC have been found to correlate with the cholesterol content in atherosclerotic lesions and the severity of coronary artery disease [7]. Patients with genetic CYP27 deficiency suffer from cerebrotendinous xanthomatosis and develop premature atherosclerosis despite having normal levels of plasma cholesterol [2]. Taken together, 27OHC has been proposed to be an anti-atherogenic molecule [2,8].

Since the classical reverse cholesterol transport is mediated by HDL, and because HDLs are important carriers of 27OHC in plasma [9], we investigated whether monogenic disorders of HDL metabolism affect plasma and lipoprotein concentrations of 27OHC. We used an LC–MS method which allows to quantify 27OHC in small plasma volumes (15–50 μL) [10]. Specifically, we analysed plasmas of patients with functionally relevant mutations or mice with knock-outs of the following genes: apoA-I which is the main protein component of HDL; ATP-binding cassette transporters A1 (ABCA1) and G1 (ABCG1) which mediate cholesterol and phospholipid efflux from cells to apoA-I and HDL, respectively; lecithin:cholesterol acyl transferase (LCAT) which esterifies cholesterol and thereby converts discoidal HDL precursors into mature spherical HDL particles; cholesterylester transfer protein (CETP, not expressed in mice) which exchanges cholesterylester of HDL against triglycerides of apoB-containing lipoproteins; scavenger receptor type B I (SR-BI) which mediates selective uptake of cholesterol esters in the liver and steroidogenic organs; and hepatic lipase (HL, only studied in humans) which hydrolyses triglyceride and phospholipids in HDL [11]. Through this analysis, we identified variations in HDL concentrations as well as the activities of ABCA1, ABCG1, LCAT and CETP as factors regulating 27OHC metabolism in plasma.

2. Methods

2.1. Patient plasmas

Forty-one Dutch patients with functionally relevant mutations in the genes encoding for either LCAT, ABCA1, APOA1, SR-BI, CETP or HL as well as 41 unaffected family members were investigated. Most of the families and the underlying defects were described previously [12–16]. In addition, 4 Danish patients with mutations in APOA-I or CETP as well as 5 age- and sex-matched controls were included. The Medical Ethics Committee of the Academic Medical Center (AMC) in Amsterdam, The Netherlands, as well as the Danish Ethics Committee for Copenhagen and Frederiksberg, Denmark, approved all genetic and phenotypic studies described and all participants signed an informed consent to join the study. The characteristics of the study participants are shown in Table 1. Fasting blood samples were collected after at least a 10-h fast in the morning and EDTA-plasma was prepared through centrifugation of the blood at 3000 rpm for 10 min at 25 °C by the study nurses of the AMC, Amsterdam, The Netherlands, and of the Department of Clinical Biochemistry, Rigshospitalet, Copenhagen, Denmark. Aliquots were immediately frozen at −80 °C until later use.

2.2. Mouse plasmas

All animal procedures were approved by the Bioethical committee of the Biomedical Research Institute of the Academy of Athens and Leiden University. All animal experimentations were in agreement with the ethical recommendation of the European Communities Council Directive (86/609/EEC).

Knock-out as well as C57B/6 control mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and maintained on standard chow diets containing 5% fat (Harlan Teklad, Madison, WI, USA). Mice were fasted for 6 h before the collection of blood samples. Samples from mutants and wild-type control mice were collected in EDTA-containing eppendorf tubes, kept in room temperature for 20 min and centrifuged to collect the plasma. All samples were stored in −80 °C and transported in dry ice.

Bone marrow transplantation was conducted by Division of Biopharmaceutics, Leiden/Amsterdam Center for Drug Research, LeidenUniversity, The Netherlands [17]. Bone marrow transplant mice as well as their controls were fed with Western diet containing 0.25% cholesterol.

2.3. Quantification of 27OHC

The concentrations of 27OHC in total and apoB-depleted plasma were analysed using a dopant assisted-atmospheric pressure photoionization (DA-APPI) liquid chromatography–mass spectrometry (LC–MS) method as described previously [10]. For the quantification of unesterified 27OHC, the hydrolysis step was omitted and the sample volume was increased to 100 μL due to the very low endogenous level of free 27OHC (only ∼10–20% of total 27OHC) [9].

For the determination of 27OHC in HDL, apoB-containing lipoproteins were removed from 100 μL human EDTA plasma by precipitation with dextran-sulfate-Mg^{2+} [18]. 27OHC concentrations assayed in apoB-depleted plasma were multiplied by 1.1 to correct for the dilution and to obtain the concentration of 27OHC in HDL. The difference between the concentrations of total 27OHC and HDL-27OHC was described as nonHDL-27OHC concentration. We have shown previously that this method recovers about 95% of 27OHC in the apoB-depleted fraction, as compared to density-gradient ultracentrifugation, and that nearly 90% of plasma 27OHC is contained in the lipoprotein fractions [9].

2.4. Quantification of other lipids, apolipoproteins, LCAT and CETP

Total plasma cholesterol, triglycerides, HDL-cholesterol, and LDL-cholesterol, apoB, apoA-I, and apoA-II levels were measured with commercial kits (Wako, Neuss, Germany and Randox, Crumlin, UK) on a Cobas Mira autoanalyzer. Due to the limited sensitivity of the enzymatic homogenous assay, concentrations of HDL cholesterol as well as thereof derived measures (nonHDL cholesterol and the ratios of 27OHC/cholesterol in HDL and nonHDL) are not reported for patients with virtually complete HDL deficiency, namely the homozygotes and compound heterozygotes for mutations in ABCA1 or LCAT.

The unesterified cholesterol concentrations in the supernatant after precipitation of apoB-containing lipoproteins with polyethylenglycol were assayed using a procedure that has been developed in house. Briefly, a mixture of reagent (200 μL) containing 7.3 mg/mL homovanilic acid, 0.1 M 3-[N-Morpholine]propanesulfonic acid, 1.25 mM taurocholate, 1.35 U/mL cholesterol oxidase and 10 U/mL peroxidase was added to 30 μL sample and assayed using the FLUOstar Galaxy fluorometer (BMG Labtech, Offenburg, Germany). The assay was based on the reaction of cholesterol with oxygen to give hydrogen peroxide, which subsequently reacted with homovanilic acid to give a fluorescence emission at 450 nm wavelength. Cholesterylester concentrations were calculated as the differences of total and unesterified cholesterol concentrations.

LCAT activity and cholesterol esterification rates were determined using proteoliposomes containing radioactive cholesterol as the exogenous substrate assay and after equilibration of plasma

Table 1
Characteristics of the mutation carriers and their unaffected family members.

Mutated gene	Number of defective alleles	Mutation[a]	Age (year)	Cholesterol (mM)	HDL-cholesterol (mM)	NonHDL-cholesterol (mM)	Triglyceride (mM)	Number of smokers
Dutch								
APOA1	0		27 ± 14	4.59 ± 0.68	1.16 ± 0.06	3.43 ± 0.63	1.09 ± 0.29	0
	1	p.L202P (c.605T > C)	26 ± 17	3.61 ± 0.31	0.51 ± 0.35	3.10 ± 0.06	1.09 ± 0.51	0
ABCA1	0		44 ± 20	4.39 ± 0.89	1.47 ± 0.39	2.92 ± 0.62	0.95 ± 0.22	1
	1	p.L1056P (c.3167T > C) or p.C1477R (c.4429T > C)	57 ± 11	4.47 ± 1.08	0.94 ± 0.17	3.53 ± 0.96	1.01 ± 0.05	0
	2	p.L1056P (c.3167T > C, homozygote) or p.Q1038X (c.3112C > T) + p.N1800H (c.5398A > C) or p.C1477R (c.4429T > C) + IVS25 + 1G > C	53 ± 10	2.89 ± 2.39	ND[b]	ND[b]	2.29 ± 1.80	0
LCAT	0		49 ± 9	4.96 ± 0.86	1.33 ± 0.38	3.62 ± 0.97	1.29 ± 0.66	0
	1	p.T147I (c.440C > T), p.R322C (c.964C > T), p.N155D (c.463A > G), p.P34Q (c.101C > A), p.Y107X (c.321C > A), p.I202T (c.605T > C) or p.V333M (c.997G > A)	43 ± 13	4.27 ± 1.21	0.81 ± 0.28	3.45 ± 1.08	1.30 ± 0.55	1
	2	p.T147I (c.440C > T) + V333M	69 ± 4	3.26 ± 0.19	ND[b]	ND[b]	2.11 ± 0.49	0
SR-BI	0		54 ± 19	4.77 ± 0.89	1.17 ± 0.33	3.60 ± 0.79	1.21 ± 0.64	0
	1	p.P297S (c.889C > T)	45 ± 22	4.46 ± 1.21	1.73 ± 0.56	2.73 ± 0.81	0.97 ± 0.28	1
CETP	0		36 ± 16	4.14 ± 0.51	1.30 ± 0.21	2.85 ± 0.48	0.87 ± 0.40	1
	1	IVS7 + 1 (G > T)	39 ± 18	4.20 ± 0.51	1.56 ± 0.29	2.64 ± 0.77	0.76 ± 0.32	1
HL (LIPC)	0		45 ± 19	5.23 ± 0.99	1.61 ± 0.54	3.62 ± 0.90	1.45 ± 1.05	3
	1	p.S289F (c.866C > T)	45 ± 15	4.92 ± 1.21	2.00 ± 0.68	2.92 ± 0.86	1.14 ± 0.43	1
Danish								
Controls	0		50 ± 9	5.84 ± 1.24	1.54 ± 0.24	4.30 ± 1.23	1.34 ± 0.62	1
APOA1	1	p.L168R (c.503T > G)	63 ± 4	4.70 ± 0.28	0.85 ± 0.07	3.85 ± 0.35	1.27 ± 0.70	0
CETP	1	p.S349Y (c.1046C > A)	59 ± 4	6.85 ± 2.05	3.05 ± 1.77	3.80 ± 0.28	0.86 ± 0.23	1

Values represent mean ± SD.
[a] Amino acid changes are localized on the basis of the entirely translated protein, that is including the signal peptides. To define the position within the mature protein, correct apoA-I by −24 amino acids, ABCA1 by −60 amino acids, LCAT by −24 amino acids, SR-BI by 0 amino acids, CETP by −17 amino acids, HL by −22 amino acids.
[b] Not determined (ND).

with ^{3}H-cholesterol as the endogenous substrate, respectively [19]. CETP concentration was measured as described [20].

2.5. Statistical analysis

Statistical analyses were performed using Microsoft Excel (Microsoft, Redmont, WA, USA) and SPSS 17.0 (SPSS, Chicago, IL, USA). Normal distribution was tested using Kolmogorov–Smirnov test. Since the data distribution was normal, unpaired student *t*-test was used to test for statistical significances of differences between 2 groups (assuming equal variance). Significance of a correlation was tested from the Pearson's correlation coefficient (*r*).

3. Results

3.1. Effects of gender, age and statin treatment on 27OHC levels in humans

Because of a strong effect of gender on plasma 27OHC levels [9], we stratified our data by sex. Because 27OHC concentrations in plasma are only mildly affected by age [9], we did not further adjust the data for age. About 35% of all probands were treated with statins. However, when comparing family controls with and without statin treatment, this did not appear to affect plasma- and HDL-27OHC concentrations: 0.47 ± 0.07 μM *vs.* 0.50 ± 0.15 μM, and 0.20 ± 0.05 μM *vs.* 0.23 ± 0.15 μM, respectively. Also other common medications including oral contraceptives, beta blockers, calcium channel blockers, angiotensin converting enzyme inhibitors as well diuretics were not associated with statistically significant differences of 27OHC concentrations in plasma, HDL or nonHDL, and we therefore did not stratify the data for statin or any other drug treatment.

3.2. Effects of human inborn errors of HDL metabolism on 27OHC levels

In Table 2, we compared the concentrations of 27OHC in total plasma, HDL and nonHDL. As shown previously [9], we identified highly significant and similar positive correlations between 27OHC and cholesterol levels in total plasma, HDL and nonHDL fractions (see Fig. 1). The *r*-square values obtained by univariate regression analysis of data indicate that variations in total cholesterol, HDL cholesterol and nonHDL cholesterol explain 35–40% of the variance in plasma concentrations of total 27OHC, HDL-27OHC and nonHDL-27OHC, respectively. The correlation plots as well as the ratios of 27OHC to cholesterol (Fig. 1 and Table 2) allowed us to identify those conditions in which 27OHC levels were altered beyond cholesterol levels.

Homozygous defects in LCAT and ABCA1 were associated with significantly increased 27OHC/cholesterol ratios in plasma (Table 2). All other differences in plasma concentration of 27OHC between carriers of other gene defects and family controls were either not statistically significant or not consistent: Male but not female SR-BI mutation carriers showed lower 27OHC plasma levels as compared to all male controls, but not as compared to unaffected

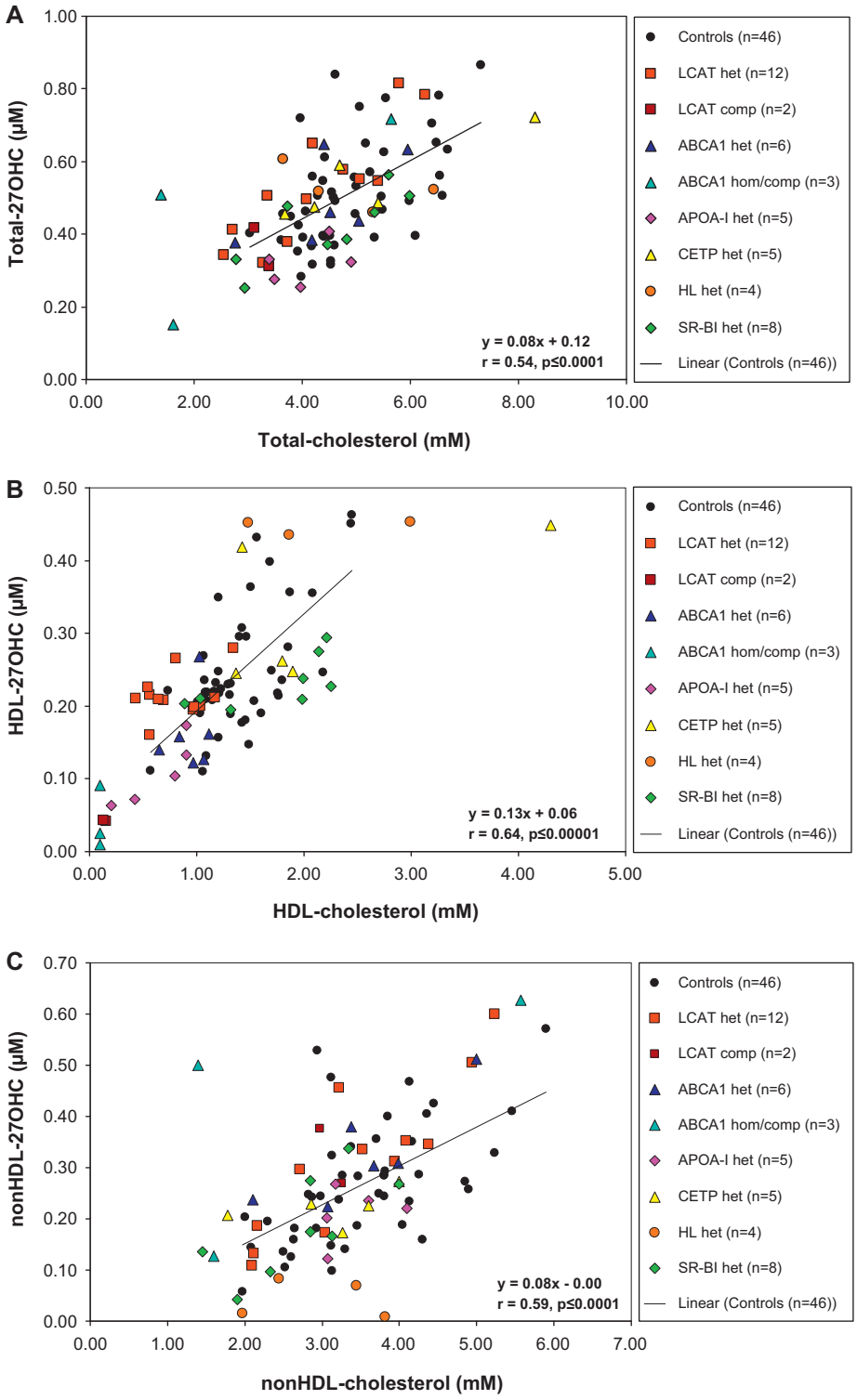

Fig. 1. Plots of 27OHC *vs.* cholesterol concentrations in plasma (A), HDL (B) and nonHDL (C) of individuals with mutations in HDL genes and their unaffected relatives. The correlations and regression equations have been calculated on data from all unaffected family members ("Controls" of Tables 1 and 2). Please note the biases towards elevated 27OHC concentrations in plasma, HDL and nonHDL of LCAT mutation carriers as well as the biases towards higher HDL-27OHC and lower nonHDL-27OHC concentrations in the carriers of HL mutations.

male relatives from the same family. The significantly decreased levels of 27OHC plasma levels in male and female carriers of the apoA-I(p.L202P) mutant were not retrieved in individuals carrying the apoA-I(p.L168R) mutation (Table 2).

Defects in LCAT and ABCA1 were associated with gene-dosage-dependent decreases of HDL-27OHC levels. Heterozygous carriers of apoA-I mutations also showed significantly decreased 27OHC levels in HDL. Conversely, defects that increase HDL-cholesterol levels also tended to be associated with an increase of HDL-27OHC levels (Table 2 and Fig. 1B). However, this effect was statistically significant only for male CETP and HL mutation carriers. After normalisation for HDL-cholesterol levels, defects in LCAT showed

Table 2
27OHC levels and 27OHC/cholesterol ratios in plasma and lipoproteins in families with monogenic disturbances of HDL metabolism.

Mutated gene	Number of defective alleles	Number of participants		27OHC in total plasma (μM)		Ratio total-27OHC (μM)/total-cholesterol (mM)		27OHC in HDL (μM)		Ratio HDL-27OHC (μM)/HDL-cholesterol (mM)		27OHC in nonHDL (μM)		Ratio nonHDL-27OHC (μM)/nonHDL-cholesterol (mM)	
		Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male
Summarized data of all unaffected family controls	0	21	24	0.43 ± 0.10	0.60 ± 0.14	0.09 ± 0.01	0.12 ± 0.02	0.24 ± 0.09	0.26 ± 0.08	0.15 ± 0.03	0.21 ± 0.04	0.19 ± 0.06	0.34 ± 0.11	0.06 ± 0.01	0.09 ± 0.03
Mutations causing decreased levels of HDL-cholesterol															
APOA1	0	6	2	0.42 ± 0.08	0.74 ± 0.18	0.08 ± 0.02	0.13 ± 0.01	0.21 ± 0.04	0.26 ± 0.04	0.15 ± 0.04	0.20 ± 0.01	0.21 ± 0.06	0.48 ± 0.14	0.06 ± 0.01	0.11 ± 0.01
p.L202P (c.605T > C)	1	2	1	0.26 ± 0.01$^{*\#}$	0.33$^{\#}$	0.07 ± 0.01	0.10	0.10 ± 0.04$^{**\#}$	0.06$^{\#}$	0.16 ± 0.02	0.31$^{\#}$	0.16 ± 0.06	0.27	0.05 ± 0.02	0.08
p.L168R (c.503T > G)	1	2	0	0.37 ± 0.06		0.08 ± 0.02		0.14 ± 0.05*		0.16 ± 0.04		0.23 ± 0.01		0.06 ± 0.01	
ABCA1	0	4	4	0.38 ± 0.09	0.50 ± 0.11	0.08 ± 0.01	0.12 ± 0.01	0.20 ± 0.04	0.23 ± 0.05	0.12 ± 0.01	0.19 ± 0.02	0.18 ± 0.05	0.27 ± 0.06	0.06 ± 0.01	0.09 ± 0.01
	1	3	3	0.40 ± 0.03	0.58 ± 0.10	0.11 ± 0.03	0.12 ± 0.02	0.14 ± 0.02	0.18 ± 0.08	0.16 ± 0.05	0.19 ± 0.07	0.26 ± 0.05	0.40 ± 0.10	0.09 ± 0.02	0.10 ± 0.02
	2	2	1	0.43 ± 0.40	0.51	0.11 ± 0.02$^{\#}$	0.36$^{***\#\#\#}$	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]
LCAT	0	3	5	0.42 ± 0.04	0.64 ± 0.13	0.09 ± 0.01	0.12 ± 0.02	0.29 ± 0.06	0.27 ± 0.06	0.17 ± 0.02	0.25 ± 0.05	0.13 ± 0.03	0.36 ± 0.09	0.05 ± 0.02	0.09 ± 0.02
	1	4	8	0.47 ± 0.15	0.56 ± 0.17	0.11 ± 0.03$^{\#}$	0.13 ± 0.02	0.20 ± 0.01*	0.22 ± 0.04	0.22 ± 0.06$^{\#\#\#}$	0.33 ± 0.10$^{\#\#\#}$	0.27 ± 0.16	0.34 ± 0.15	0.08 ± 0.04$^{\#}$	0.09 ± 0.02
	2	2	0	0.37 ± 0.07		0.11 ± 0.03$^{\#}$		ND[a]		ND[a]		ND[a]		ND[a]	
Mutations causing increased levels of HDL-cholesterol															
SR-BI	0	4	4	0.42 ± 0.11	0.55 ± 0.15	0.08 ± 0.01	0.12 ± 0.04	0.17 ± 0.06	0.19 ± 0.05	0.13 ± 0.03	0.19 ± 0.01	0.25 ± 0.06	0.37 ± 0.13	0.07 ± 0.01	0.11 ± 0.05
	1	5	3	0.42 ± 0.09	0.41 ± 0.14$^{\#}$	0.09 ± 0.02	0.10 ± 0.02	0.24 ± 0.04	0.22 ± 0.02	0.12 ± 0.02	0.18 ± 0.06	0.18 ± 0.09	0.19 ± 0.13$^{\#}$	0.07 ± 0.03	0.06 ± 0.04
CETP	0	5	3	0.43 ± 0.09	0.76 ± 0.17	0.09 ± 0.02	0.14 ± 0.03	0.22 ± 0.03	0.30 ± 0.06	0.15 ± 0.03	0.22 ± 0.02	0.20 ± 0.07	0.46 ± 0.12	0.06 ± 0.02	0.12 ± 0.03
(IVS7 + 1 (G > T))	1	1	2	0.45	0.53 ± 0.08	0.12	0.12 ± 0.01	0.25	0.33 ± 0.12$^{\#}$	0.13	0.24 ± 0.08	0.21	0.20 ± 0.04	0.12$^{\#\#\#}$	0.07 ± 0.02
p.S349Y (c.1046C > A)	1	1	1	0.49	0.72	0.09	0.09	0.26	0.45$^{\#}$	0.15	0.10$^{*\#\#}$	0.22	0.27	0.06	0.08
HL (LIPC)	0	3	7	0.51 ± 0.17	0.58 ± 0.12	0.09 ± 0.02	0.11 ± 0.01	0.36 ± 0.16	0.29 ± 0.10	0.17 ± 0.04	0.21 ± 0.04	0.15 ± 0.04	0.29 ± 0.11	0.05 ± 0.00	0.07 ± 0.02
	1	1	3	0.52	0.53 ± 0.07	0.08	0.12 ± 0.04	0.45$^{\#}$	0.49 ± 0.08$^{**\#\#\#}$	0.15	0.30 ± 0.06$^{**\#\#\#}$	0.07	0.04 ± 0.04$^{**\#\#\#}$	0.02$^{*\#\#}$	0.01 ± 0.02$^{**\#\#\#}$

Controls, sum of all unaffected family members ("0"); "0", unaffected family members; "1", heterozygous mutation carriers; "2", homozygous or compound heterozygous mutation carriers. *, **, *** and $^{\#}$, $^{\#\#}$, $^{\#\#\#}$ indicate statistically significant differences ($p < 0.05$, $p < 0.01$, $p < 0.001$, respectively) as compared to "0" and controls, respectively.

[a] Not determined (ND).

consistent and statistically significant associations with increased HDL-27OHC/HDL-cholesterol ratios (Table 2).

Finally, 27OHC levels and the ratio of 27OHC/cholesterol were increased in the nonHDL fractions of ABCA1 mutation carriers and female LCAT mutation carriers (Table 2 and Fig. 1C). By contrast, male heterozygotes for defects in apoA-I, SR-BI, CETP or HL had lower nonHDL-27OHC levels as compared to the unaffected male controls (Table 2). In HL mutation carriers, nonHDL-27OHC levels and nonHDL-27OHC/nonHDL-cholesterol ratio were lower than in unaffected controls from either the same families or the entire population (Table 2 and Fig. 1C).

3.3. Effects of LCAT and CETP mutations on esterified 27OHC in humans

LCAT activity was decreased to about 75% of normal in female and male heterozygotes for LCAT defects (Table 3). This 25% decrease in LCAT activity affected neither the esterification nor the cholesterylester/total cholesterol ratios in total plasma and HDL. By contrast, the percentage of 27OHC-ester was significantly decreased while the ratio of free-27OHC/free-cholesterol was significantly increased in both plasma and HDL of heterozygous LCAT mutation carriers as compared to unaffected family members. Regression analysis revealed a stronger correlation of LCAT activity with 27OHC-ester concentration than with cholesterylester concentration in HDL ($r = 0.6528$, $p \leq 0.001$ *vs.* $r = 0.0538$, $p \leq 0.01$, respectively; see Supplemental Fig. 1). Interestingly, the percentage of esterified 27OHC was also significantly decreased in plasma and HDL of heterozygous apoA-I(p.L202P) carriers, but not of heterozygotes for the apoA-I(p.L168R) mutation. This finding is surprising because both *in vitro* and *in vivo* the p.L168R substitution rather than the p.L202P substitution was found to reduce the LCAT cofactor activity of apoA-I (*V.I. Zannis, A. Tybjaerg Hansen, J. Albert Kuivenhoven; personal communication*). No significant changes were observed in the degree of 27OHC-esterification in carriers of ABCA1 defects.

Heterozygosity for the Dutch CETP mutations was associated with decreased absolute and relative concentrations of 27OHC-esters in the nonHDL fraction but no differences in the HDL fraction (Table 3). Despite half normal CETP concentration and CETP activity [16] the concentrations and percentages of cholesterylesters in HDL or nonHDL were not affected. Regression analysis revealed a significant correlation of CETP mass concentration with both absolute and relative 27OHC-ester concentrations in nonHDL ($r^2 = 0.337$, $p = 0.0092$ and $r^2 = 0.276$, $p = 0.029$, respectively), but insignificant correlations with absolute and relative cholesterylester concentrations in nonHDL ($r^2 = 0.077$ and $r^2 = 0.029$, respectively) (see Supplemental Fig. 2).

3.4. 27OHC in mice with various knock-outs of HDL genes

In normal murine plasma both cholesterol and 27OHC are almost exclusively carried by HDL (Supplemental Table). We therefore analysed the different genetic mouse strains for total 27OHC plasma levels only. Compared to wild-type mice, apoA-I- or LCAT-knockout mice were characterized by more than 50% lowered plasma 27OHC concentrations, whereas SR-BI knockout mice had about 5-fold higher 27OHC levels. After normalization for total cholesterol, the differences remained statistically significant for LCAT and SR-BI knock-out mice only. In addition and compared to wild type mice, ABCA1 deficient mice showed a 140% increased 27OHC/cholesterol ratio ($p < 0.001$) in the presence of 25% lower 27OHC levels ($p < 0.05$, Table 4). Interestingly, heterozygous ABCA1 knock-out mice showed a similar decrease in 27OHC plasma levels which did not result in a significantly altered 27OHC/cholesterol ratio (Table 4) due to the equal decrease in cholesterol levels.

Table 3
Esterification of 27OHC and cholesterol in plasma and lipoproteins of LCAT and CETP mutation carriers and their unaffected family controls.

	LCAT mutation carriers		CETP mutation carriers	
	Unaffected family members ("0")	Heterozygous mutants ("1")	Unaffected family members ("0")	Heterozygous mutants ("1")
Number (male/female)	5/3	8/4	2/1	2/1
LCAT activity (nmol cholesteryl ester/h × mL)	8.37 ± 1.21	6.58 ± 1.01***	N.D.	N.D.
CETP mass (μg/mL)	2.12 ± 0.40	1.73 ± 0.32*	1.93 ± 0.33	0.90 ± 0.21**
% 27OHC-ester in plasma	92.3 ± 6.7	89.8 ± 4.3	83.8 ± 4.3	75.4 ± 4.0
% cholesteryl ester in plasma	87.7 ± 2.4	88.4 ± 1.4	67.6 ± 0.6	66.7 ± 1.1
Ratio 27OHC-ester (μM)/cholesteryl ester (mM) in plasma	0.16 ± 0.04	0.16 ± 0.03	0.18 ± 0.08	0.14 ± 0.01
Ratio unesterified 27OHC (μM)/unesterified cholesterol (mM) in plasma	0.09 ± 0.06	0.15 ± 0.08	0.07 ± 0.01	0.09 ± 0.02
% 27OHC-ester in HDL	88.4 ± 4.4	79.8 ± 10.6*	78.5 ± 13.5	81.9 ± 2.6
% cholesterol esters in HDL	80.9 ± 3.0	82.5 ± 3.6	77.9 ± 2.3	75.7 ± 2.4
Ratio 27OHC-ester (μM)/cholesteryl ester (mM) in HDL	0.22 ± 0.06	0.21 ± 0.07	0.27 ± 0.10	0.28 ± 0.11
Ratio unesterified 27OHC (μM)/unesterified cholesterol (mM) in HDL	0.12 ± 0.04	0.28 ± 0.20*	0.23 ± 0.07	0.21 ± 0.11
% 27OHC-ester in nonHDL	93.3 ± 23.9	95.8 ± 9.6	88.9 ± 5.7	67.0 ± 9.6*
% cholesterol esters in nonHDL	92.5 ± 1.4	91.4 ± 2.0	64.5 ± 1.0	63.4 ± 1.1
Ratio 27OHC-ester (μM)/cholesteryl ester (mM) in nonHDL	0.13 ± 0.06	0.14 ± 0.05	0.14 ± 0.04	0.07 ± 0.01
Ratio unesterified 27OHC (μM)/unesterified cholesterol (mM) in nonHDL	0.14 ± 0.19	0.10 ± 0.10	0.03 ± 0.02	0.07 ± 0.04

*, ** and *** indicate statistically significant differences ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively) between "1" and "0".

To investigate the contribution of macrophage-derived 27OHC to plasma 27OHC levels, we also analysed the plasmas of LDL receptor-deficient mice who received bone marrow from mice with knockouts of either ABCA1, ABCG, or both ABCA1 and ABCG1 (Table 4). Notably in parallel with hypercholesterolemia, LDLR−/− mice had 20-fold higher 27OHC concentrations and 2-fold higher 27OHC/cholesterol ratios than wild-type mice. Transplantation of ABCA1- or ABCG1-deficient bone marrow resulted in 45% and 23% decreases of 27OHC plasma levels as well as in significantly decreased 27OHC/cholesterol ratios ($p < 0.05$ for ABCA1; $p < 0.01$ for ABCG1). Mice with bone marrow from mice with knock-outs of both ABCA1 and ABCG1 had even 66% lower mean 27OHC plasma levels, however, this was seen in the context of a slightly more pronounced 70% decrease in cholesterol levels so that the 27OHC/cholesterol ratio was rather increased.

4. Discussion

27OHC plays an important physiological role as an intermediary product of cholesterol removal from specific tissues and the entire organism as well as an agonist and modulator of liver-X- and estrogen receptors, respectively [9,21]. We have previously shown for normolipidemic individuals that about 50%, 30% and 5% of circulating 27OHC in healthy individuals are recovered in HDL, LDL and VLDL, respectively [9]. However, the plasma concentration of 27OHC is 10,000-fold lower than that of cholesterol (0.5 μmol/L *vs.* 5 mmol/L on average). It is even considerably lower than the particle concentration of HDL (on average 20 μmol/L) and LDL (on average 2 μmol/L) so that only a minority of lipoprotein particles carries 1 or more molecules of 27OHC. The very low plasma and lipoprotein concentrations of 27OHC, the significant correlations between 27OHC and cholesterol concentrations in plasma and lipoproteins [9] as well as the higher water-solubility of 27OHC compared to cholesterol [3] raised the question whether 27OHC is passively equilibrated among the various lipoprotein classes or specifically metabolized in plasma similar to cholesterol, that is by established transporters, enzymes, lipid transfer proteins and receptors. Some general support for the latter assumption could already be derived from the enrichment of 27OHC relative to cholesterol in HDL (on average 47 ± 7% of plasma 27OHC instead of 33 ± 6% plasma cholesterol) [9] and from the 10% higher percentages of 27OHC-esters relative to cholesterylesters in plasma, HDL and LDL [9]. We here further explored this question by analysing 27OHC concentrations in plasma as well as HDL- and nonHDL-fractions of patients and mice with distinct monogenic disorders of HDL metabolism.

In the present study correlations between 27OHC and cholesterol concentrations in plasma and HDL were similar or even

Table 4
27OHC plasma levels in mice with knock-outs of LCAT (LCAT−/−), APOA-I (APOA1−/−), ABCA1 (ABCA1−/−) or SR-BI (SRBI−/−) as well as LDL receptor deficient mice (LDLR−/−) which received bone-marrow from either wild type mice (LDLR−/−:BMT^{wt}) or mice with knock-outs of either ABCA1 (LDLR−/−:BMT$^{ABCA1-/-}$), ABCG1 (LDLR−/−:BMT$^{ABCG1-/-}$) or both ABCA1 and ABCG1 (LDLR−/−:BMT$^{ABCA1-/- \times ABCG1-/-}$).

Mouse strains	Number of mice (n)	27OHC (μmol/L)	Cholesterol (mmol/L)	Ratio total-27OHC/total-cholesterol (μmol/mmol)
Wild type	28	0.15 ± 0.08	1.81 ± 0.55	0.08 ± 0.04
APOA-I−/−	5	0.06 ± 0.04*	1.24 ± 0.43***	0.05 ± 0.05
ABCA1−/−	6	0.11 ± 0.06	0.68 ± 0.50***	0.19 ± 0.08***
ABCA1+/−	3	0.08 ± 0.01	1.43 ± 0.21	0.06 ± 0.01
LCAT−/−	8	0.06 ± 0.02**	2.28 ± 1.08	0.03 ± 0.01***
SR-BI−/−	10	0.96 ± 0.32***	3.27 ± 0.73***	0.29 ± 0.10***
LDLR−/−:BMT ^{wt}	6	3.33 ± 1.32	20.44 ± 7.83	0.16 ± 0.03
LDLR−/−:BMT$^{ABCA1-/-}$	6	1.88 ± 0.26*	15.56 ± 2.89	0.13 ± 0.03*
LDLR−/−:BMT$^{ABCG1-/-}$	6	2.57 ± 0.52	23.86 ± 3.02	0.11 ± 0.03**
LDLR−/−:BMT$^{ABCA1-/- \times ABCG1-/-}$	9	1.11 ± 0.25***	6.09 ± 1.55***	0.19 ± 0.07

*, **, and *** indicate statistically significant differences ($p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively) as compared to wild type mice or LDLR−/− mice transplanted with wild type bone marrow.

stronger than those reported by us previously, namely 0.59 instead of 0.54 in total plasma, and 0.64 instead of 0.34 in HDL (Fig. 1 and [9]). Independently of the metabolic or genetic origin, differences in HDL cholesterol appear to explain up to 40% of the variation in HDL-27OHC levels. As a consequence, functionally relevant mutations in apoA-I, ABCA1 and LCAT that decrease HDL-cholesterol were associated with reduced concentrations of 27OHC in HDL. Monoallelic and biallelic mutations, which could only investigated for ABCA1 and LCAT, led to gene-dose-dependent decreases of HDL-27OHC levels. Likewise, HDL deficient mice with knock-outs of apoA-I or LCAT presented with decreased 27OHC plasma levels. Conversely, 27OHC or HDL-27OHC plasma levels were strongly elevated in SR-BI knock-out mice as well as in male carriers of mutations in the genes of CETP, SR-BI and HL. The reason for the absent effect of the HDL-cholesterol increasing mutations on 27OHC levels in women might be related to the strong regulation of HL and SR-BI by estrogens [22,23], since 27OHC is a modulator of estrogen receptor activity [9,21]. One may speculate that this interaction of 27OHC and estrogens in the regulation of SR-BI and HL leads to differences in the metabolism of cholesterol and 27OHC between males and females.

At first sight, in both humans and mice with inborn errors of HDL metabolism, HDL-27OHC levels appeared simply to change passively in parallel with HDL-cholesterol levels. However, in the light of the previously suggested concept that 27OHC may represent an HDL-independent pathway of reverse cholesterol transport [2] our findings in patients with defects in ABCA1, LCAT or CETP as well as mice with either systemic or macrophage specific knockouts of ABCA1 and ABCG1 deserve special attention.

4.1. ABCA1 and ABCG1

27OHC secretion has been suggested to represent an alternative pathway to HDL- and apoA-I-mediated cholesterol efflux by which macrophages protect themselves from cholesterol overload [2]. However, this hypothesis was generated based on experiments that had been performed before the discovery of ABCA1 and ABCG1 as important cholesterol efflux pumps and their involvement in 27OHC efflux has not yet been tested directly. ABCA1 and ABCG1 were however shown to mediate the efflux of two other oxysterols, namely 25-hydroxycholesterol and 7-ketocholesterol, respectively [24,25]. The current data show normal 27OHC plasma levels in the presence of decreased cholesterol levels and hence increased 27OHC/cholesterol ratios in both ABCA1 deficient Tangier patients and ABCA1 knock-out mice suggesting that 27OHC is secreted independently from ABCA1. However, transplantation of bone marrow from ABCA1- or ABCG1-knock out mice significantly decreased 27OHC/cholesterol ratios. The mice which received bone marrow of mice with a combined knock-out of ABCA1 and ABCG1 showed the most prominent decrease of both 27OHC and cholesterol levels so that the 27OHC/cholesterol ratio was not decreased. Nevertheless, we interpreted the data as an indication that both ABCA1 and ABCG1 contribute to 27OHC-efflux from macrophages. Our contradiction to the concept of an HDL-independent reverse cholesterol transport pathway mediated by 27OHC in humans is also supported by the enrichment of 27OHC relative to cholesterol in HDL, the strong correlation between HDL-cholesterol and HDL-27OHC in the normolipidemic human population as well as the parallel changes of HDL-cholesterol and HDL-27OHC observed in almost all human heterozygotes for HDL-related gene defects. Although not proven by us directly, our findings rather suggest that 27OHC secretion involves HDL-mediated efflux pathways that are supported by ABCA1 or ABCG1 [26].

4.2. LCAT

In both genders of humans, mutations in LCAT caused relatively less pronounced decreases in HDL-27OHC than HDL-cholesterol levels in comparison with unaffected family members, the entire control population as well as other genetic low HDL conditions. This finding is surprising because about 90% of 27OHC in normal HDL but only about 80% of 27OHC in HDL of heterozygous LCAT mutation carriers is esterified. The elevated 27OHC/cholesterol ratio in LCAT mutation carriers is hence the result of a relatively high concentration of unesterified 27OHC, rather than of esterified 27OHC. It thus appears that reduced LCAT activity increases the capacity of HDL particles to accommodate unesterified 27OHC, for example as the consequence of a higher content in phosphatidylcholine or an increased number of small HDL particles.

In both plasma and HDL, the proportions of 27OHC-esters in total 27OHC are about 10% higher than the proportions of cholesterylesters in total cholesterol (Table 3 and [9]). Moreover, heterozygous LCAT mutation carriers showed reduced 27OHC-ester/total-27OHC ratios, but normal cholesterylester/total cholesterol ratios, in HDL. Finally, LCAT activity showed a stronger correlation with 27OHC-ester concentrations than with cholesterylester concentrations in HDL ($r^2 = 0.426$, $p \leq 0.001$ *vs.* $r^2 = 0.290$, $p \leq 0.01$, respectively; see Supplemental Fig. 1). Together the data thus suggest that 27OHC is more readily esterified by LCAT and more sensitive to impairments of LCAT activity than cholesterol. Our observational data do not allow any conclusion on the biochemical basis for the higher sensitivity of 27OHC towards LCAT. One reason could be differences in the reaction kinetics (affinity and/or maximal reaction velocity) of LCAT towards 27OHC and cholesterol, so that reduced LCAT activity in mutation carriers affects 27OHC-esterification more strongly than cholesterol esterification. Importantly, 27OHC has a second free hydroxyl-group in the side chain in addition to the 3β-hydroxyl-group of the sterol which, by contrast to free hydroxyl-groups of other oxysterols, can also be esterified by LCAT [27]. Therefore, in theory, the stronger impact of LCAT activity on 27OHC-esterification may reflect the abundance of different 27OHC-ester species. Unfortunately our method did not allow for a direct and differentiated quantification of 27OHC-esters. We rather estimated the concentrations of all 27OHC-esters from the differences between total and unesterified 27OHC which were measured after and before saponification, respectively. We therefore cannot rule out that the higher concentration of 27OHC-esters relative to cholesterol esters in normal controls reflects the abundance of additional 27OHC-ester species and that the decreased 27OHC-ester formation in LCAT mutation carriers results from a specific loss of function towards the 27-hydroxyl-group. However, LCAT was reported to esterify the 27-hydroxyl-group only after esterification of the 3β-hydroxyl-group, so that 27OHC-esters were postulated to occur as either 3β-hydroxyl-monoesters or 3β/27-dihydroxyl esters only [27]. If so, our finding of decreased 27OHC-esters in heterozygous LCAT mutation carriers cannot be explained by a selectively reduced activity to esterify the 27-hydroxyl-group.

4.3. CETP

Both in the nonHDL fraction (this study) and LDL [9], more than 90% of 27OHC is esterified. To our knowledge the origin of these esters has not yet been explored directly. In analogy to cholesterylesters, 27OHC-esters of apoB containing lipoproteins may originate either from intrahepatic production by acyl-CoA:cholesterol acyltransferase (ACAT) and subsequent secretion together with VLDL [28,29] or from LCAT-mediated production either directly on apoB-containing lipoproteins or indirectly on HDL and subsequent CETP mediated transfer. ACAT has been shown

to esterify most oxysterols with much less efficacy than cholesterol [28] but its contribution to 27OHC-esterification has not been explored. Unlike in HDL, the percentage of 27OHC-esters in non-HDL was not altered by heterozygous defects in LCAT suggesting that LCAT does not play a rate limiting role for 27OHC-ester formation in LDL. Alternatively, since many of the LCAT mutants analysed by us were found in patients with fish-eye disease [14,15,19], these mutants may have retained beta-LCAT activity, that is the ability to esterify cholesterol in apoB-containing lipoproteins [14,15,19]. Most interestingly and informative, however, heterozygosity for CETP defects was associated with reduced absolute and relative concentrations of 27OHC-esters in the non-HDL fraction but normal concentrations in HDL. This suggests that CETP mediates the transfer of 27OHC-esters between HDL and apoB-containing lipoproteins. This binding and transport capacity of CETP has not yet been proven directly for 27OHC-esters but for 25-hydroxycholesterol esters [30]. Compared to cholesterylester transfer, the efficacy of 27OHC-ester transfer appears to be even more sensitive to changes in CETP activity because apoB-containing lipoproteins of heterozygous CETP mutation carriers contained normal concentrations and percentages of cholesterylesters. Furthermore, CETP mass concentrations showed statistically significant correlations with absolute and relative concentrations of 27OHC-esters in nonHDL but no significant correlations with either absolute or relative cholesterylester concentrations in nonHDL (see Supplemental Fig. 2).

In conclusion, mutations in several HDL genes lead to general alterations in the quantity of 27OHC in HDL and plasma as well as specific changes in the esterification and lipoprotein distribution of 27OHC. In general, changes in the concentration of HDL cholesterol are paralleled by equidirectional changes of HDL-27OHC levels indicating that HDL acts as a passive acceptor and transporter of 27OHC and to explain up to 40% of the variation in HDL-27OHC levels. More specifically our data from mice with macrophage specific knock out of ABCA1 and ABCG1 refutes the notion that 27OHC is effluxed from macrophages by HDL-independent pathways. In addition esterification and transfer of 27OHC (esters) appear to be more sensitive towards changes in the activities of LCAT and CETP than cholesterol. It hence appears that reverse transport of 27OHC is even more strongly influenced by HDL metabolism than cholesterol. In view of the role of 27OHC as a tissue specific modulator of transcription factors, future studies will have to show whether these specific aspects in the plasma metabolism and transport of 27-hydroxycholesterol have implications for health and disease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2010.10.042.

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