patient-oriented research

Common ABCA1 variants, HDL levels, and cellular cholesterol efflux in subjects with familial low HDL[S]

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Abstract HDL promotes cholesterol efflux from peripheral cells via ABCA1 in the first step of reverse cholesterol transport (RCT). We investigated whether the early steps of RCT were disturbed in subjects with familial low HDL and an increased risk for early atherosclerosis. Cholesterol efflux from monocyte-derived macrophages to lipid-free apolipoprotein A-I (apoA-I; %) was measured in 22 patients with familial low HDL without Tangier disease mutations and in 21 healthy controls. In addition, we defined the different alleles of *ABCA1* using single-nucleotide polymorphism haplotypes and measured *ABCA1* and *ABCG1* mRNA transcript levels in cholesterol-loaded macrophages. Similar ABCA1-mediated cholesterol efflux levels were observed for macrophages derived from control subjects and from low-HDL subjects. However, when efflux of cholesterol was estimated as cholesterol efflux to apoA-I (%)/relative *ABCA1* mRNA expression level, cholesterol removal was significantly (P = 0.001) lower in the low-HDL group. Cholesterol-loaded macrophages from low-HDL subjects showed significantly increased levels of *ABCA1* mRNA but not of *ABCG1* mRNA and were more often carriers of the rare *ABCA1* alleles L158 and R219K. These results suggest that defective ABCA1 function in cholesterol-loaded macrophages is one potential contributor to the impaired RCT process and the increased coronary heart disease risk in subjects with familial low HDL.—Soro-Paavonen, A., J. Naukkarinen, M. Lee-Rueckert, H. Watanabe, E. Rantala, S. Soderlund, A. Hiukka, P. T. Kovanen, M. Jauhiainen, L. Peltonen, and M-R. Taskinen. Common ABCA1 variants, HDL levels, and cellular cholesterol efflux in subjects with familial low HDL. *J. Lipid Res.* 2007. 48: **1409–1416.**

Supplementary key words ATP binding cassette transporter A1 • high density lipoprotein • ABCG1

The principal antiatherogenic function of HDL is its ability to promote the efflux of cholesterol from peripheral cells and transport it to the liver for excretion, process termed reverse cholesterol transport (RCT) (1). This concept was further confirmed once a homozygous defect in *ABCA1* [Mendelian Inheritance in Man (MIM) 205400 and 600046] was discovered to cause Tangier disease, a condition characterized by a virtual absence of HDL in plasma and a resultant accumulation of cholesteryl esters within cells (2–4). The ABCA1 protein mediates the efflux of phospholipids and unesterified cholesterol from peripheral cells to HDL. A lipid-poor apolipoprotein A-I (apoA-I)-phospholipid complex, the preβ-HDL particle, is the most effective cholesterol acceptor in ABCA1-mediated efflux (5). The activity of ABCA1 in the liver is the major contributor to the generation of HDL, accounting for ~80% of the circulating HDL reserve (6, 7). Based on these estimations of the contribution of liver and intestine to the HDL plasma pool, it can be stated that the monocyte/macrophage ABCA1 only minimally affects plasma HDL levels (8). Therefore, hepatic ABCA1 is critical in maintaining the circulation of mature HDL particles by direct lipidation of lipid-poor apoA-I, as it slows the HDL catabolism in the kidney and prolongs its residence time in plasma.

In subjects heterozygous for a defective allele of the *ABCA1* gene, cholesterol efflux is decreased to approximately half of that in normal individuals (9, 10). The heterozygotes also have increased carotid artery intima-

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[S] The online version of this article (available at http://www.jlr.org) contains supplemental data in the form of two tables.

media thickness (IMT) and low high density lipoprotein-cholesterol (HDL-C) and thus are at increased risk for the development of early atherosclerosis (11). The association between *ABCA1* variants and HDL-C levels was initially reported in French-Canadian families with familial hypoalphalipoproteinemia (3) and subsequently in Dutch, North American, and Danish study cohorts (9, 12–14). Recently, it was shown that up to 10% of people in the lowest HDL-C percentile are heterozygous for rare mutations in *ABCA1* (14). However, reports also exist of the lack of association between *ABCA1* variants and the level of HDL-C (15–17).

In addition to *ABCA1*, macrophages also express other *ABC* transporters such as *ABCG1* (18, 19). In contrast to *ABCA1*, which interacts with the lipid-poor preβ-HDL, *ABCG1* transports cellular cholesterol predominantly to the large spherical HDL_2 and HDL_3 particles (19–21). It is likely that a synergistic action of *ABCA1* and *ABCG1* is needed for an effective RCT process and the resultant protection against atherosclerosis (22).

The deleterious clinical phenotype of patients with low levels of HDL-C could be the result of decreased cholesterol efflux from peripheral cells to HDL, a defect of nascent HDL as a cholesterol acceptor, or a combination of both. We analyzed the efficiency of cholesterol efflux using as a model monocyte-derived macrophages obtained from Finnish subjects with familial low HDL and increased risk for premature coronary heart disease (CHD). We hypothesized that *1*) subjects with familial low HDL have reduced cellular cholesterol efflux via *ABCA1* and *2*) specific risk alleles of the *ABCA1* gene may contribute to the altered function, which in turn would be apparent at the transcript level as an aberrant expression of *ABCA1* during cholesterol loading and unloading. The levels of *ABCG1* (MIM 603076) mRNA expression were also measured to examine whether the transcript levels of the two cholesterol transporter genes would suggest coregulation, implying a shared regulatory mechanism.

SUBJECTS AND METHODS

Study subjects

Our study cohort comprised affected family members from carefully characterized Finnish low-HDL pedigrees (17) and a control group including healthy volunteers and spouses with no CHD or lipid abnormalities. The low-HDL families were ascertained based on HDL-C level below the 10th age-/sex-specific Finnish population percentile (<0.9 mmol/l for men and <1.1 mmol/l for women) in at least two first degree family members, as described previously in detail (17). Each subject gave written informed consent before participating in the study. The ethics committee of the Helsinki University Central Hospital approved the study design, and all samples were collected in accordance with the Helsinki Declaration.

The study comprised two cohorts. First, functional efflux studies were performed using cultured macrophage foam cells derived from 43 male subjects: 22 low-HDL subjects and 21 control subjects. The clinical and biochemical characteristics of the study populations participating in the efflux study are shown in **Table 1**. Second, genotyping of single-nucleotide polymorphisms (SNPs) using the array technology was carried out in 72 individuals (including those participating in the efflux study): 28 affected family members with low HDL (of whom 6 were women), 19 unaffected family members (of whom 12 were women), and 25 control subjects (of whom 4 were women). The clinical and biochemical parameters of the 72 individuals are provided in supplementary Table I.

Analytical methods

Fasting levels of lipids, apolipoproteins, glucose, and insulin were measured by standardized techniques as described previ-

TABLE 1. Clinical and biochemical characteristics of the low-HDL subjects and controls

Characteristic	Low-HDL Subjects (n = 22)	Control Subjects (n = 21)	P^a	P^b
Age (years)	52.7 ± 9.6	50.4 ± 11.8	NS	NS
Mean IMT (mm)	1.02 ± 0.21	0.86 ± 0.17	0.007	NS
BMI (kg/m^2)	28.2 ± 4.0	24.5 ± 2.7	0.001	0.003
Waist (cm)	103 ± 11	89 ± 8	<0.001	0.001
Systolic blood pressure (mmHg)	129 ± 13	133 ± 19	NS	NS
Diastolic blood pressure (mmHg)	81 ± 10	83 ± 9	NS	NS
HDL-C (mmol/l)	0.78 ± 0.16	1.54 ± 0.38	<0.001	<0.001
ApoA-I (mg/dl)	99 ± 12	139 ± 17	<0.001	<0.001
ApoA-II (mg/dl)	32 ± 6	36 ± 8	0.022	NS
Preβ-HDL (%)	13 ± 4	14 ± 4	NS	NS
Preβ-HDL mass (mg/dl)	12.6 ± 4.0	19.2 ± 7.0	0.002	0.022
HDL particle size (Å)	87.2 ± 2.4	93.4 ± 4.4	<0.001	<0.001
TG (mmol/l)	2.02 ± 1.20	1.05 ± 0.48	<0.001	0.006
TC (mmol/l)	4.34 ± 0.79	5.36 ± 0.70	<0.001	NS
LDL-C (mmol/l)	2.65 ± 0.83	3.34 ± 0.81	0.009	NS
CETP activity (nmol/ml/h)	35.3 ± 7.2	30.4 ± 4.5	0.012	0.037
PLTP activity (nmol/ml/h)	6,206 ± 1,452	6,255 ± 864	NS	NS
PLTP mass (μg/ml)	4.64 ± 1.13	6.55 ± 1.55	<0.001	0.004

ApoA-I, apolipoprotein A-I; BMI, body mass index; CETP, cholesteryl ester transfer protein; HDL-C, high density lipoprotein-cholesterol; IMT, intima-media thickness; PLTP, phospholipid transfer protein; TC, total cholesterol; TG, triglyceride. Data are presented as means ± SD.

[a] ANOVA (two-sided) *P* value.

[b] ANOVA (two-sided) *P* value with adjustment for statin use.

ously (17, 23). Preβ-HDL was quantified by crossed immunoelectrophoresis (24, 25) and expressed as a percentage of the sum of α-HDL and preβ-HDL areas. The preβ-HDL mass is expressed in absolute amount (mg/dl apoA-I present in preβ-HDL in serum). HDL particle size was determined by native gradient gel electrophoresis (26). Cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP) activities were measured as described previously (23), and PLTP mass was measured using the PLTP ELISA method (27).

DNA was extracted from blood samples and used for genotyping of SNPs using the array technology (28). SNPs were chosen by interrogating the HapMap data and other public databases and on the basis of the polymorphisms identified in our earlier sequencing effort (17). All genotyped SNPs were in Hardy-Weinberg equilibrium.

Measurement of apoA-I-mediated cholesterol efflux from cultured macrophages

Human monocyte-derived macrophages were obtained from human whole blood by cell culturing. Fasting blood (60 ml) was drawn into tubes containing citrate as an anticoagulant. Buffy coat was promptly separated by low-speed centrifugation (1,500 *g*) at room temperature. Diluted buffy coat was then layered over Ficoll-Paque and centrifuged again (2,000 *g*, 40 min), and the mononuclear cells were recovered as a cell layer. Subsequently, three washes with PBS were performed to eliminate platelets from the mononuclear pellet. Finally, the cell pellet was suspended in DMEM. Mononuclear cells were plated onto 24-well plates (2 million cells per well) and allowed to stay in the wells for 1 h for attachment, after which macrophage medium with Macrophage Colony-stimulating Factor (M-CSF) was added. The medium was then changed every 2–3 days. After 7 days, when the monocytes had been phenotypically converted to macrophages, they were labeled with [^{3}H]cholesteryl linoleate by incubating them for 48 h with 25 μg/ml [^{3}H]cholesteryl linoleate-acetyl-LDL. This loading procedure induced the formation of a cellular pool of radioactively labeled cholesteryl esters. To induce ABCA1-dependent cholesterol efflux from the radiolabeled macrophages, purified human lipid-free apoA-I (10 μg/ml; kindly provided by Dr. Peter Lerch of the Swiss Red Cross) was added to the medium as cellular cholesterol acceptor (29). After incubation for 16 h, an interval within which efflux is linear (30), incubation medium was collected. Radioactivity in the medium and in washed cells was determined by scintillation counting, and the fractional cholesterol efflux (as percentage) was calculated as $dpm_{medium}/(dpm_{cell} + dpm_{medium}) \times 100$. Efflux values to incubation medium in the absence of apoA-I were subtracted from those in the presence of apoA-I.

Expression analyses

Monocyte-derived macrophages were collected for mRNA extraction *1*) after cholesterol loading, *2*) after efflux in the absence of apoA-I, and *3*) after efflux in the presence of apoA-I. Macrophage mRNA was extracted and quantified as described previously (31). Quantitative PCR, using the SYBR-Green assay (Applied Biosystems), was done to measure the relative abundance of transcripts. Two-step RT-PCR was done using the TaqMan Gold RT-PCR kit. Primer sequences are available on request. To compare the relative *ABCA1* and *ABCG1* expression levels at different stages, the relative mRNA expression in each experiment was normalized against the expression level of the housekeeping gene *GAPDH*. The reliability of *GAPDH* as a housekeeping gene was evaluated by studying the expression of *GAPDH* in a sample of 47 fat biopsies assayed on Affymetrix U133 Plus 2.0 chips according to standard protocols. Samples were obtained from individuals from the same dyslipidemic families as the monocyte-derived macrophages used in this study. Correlation of housekeeping genes to any possible confounders, such as age, sex, body mass index (BMI), HDL, total cholesterol (TC), and triglyceride (TG), was evaluated by the Pearson correlation using SPSS 11.0 statistical software (SPSS, Inc., Chicago, IL). No correlation to any of these variables could be identified for *GAPDH* expression, and the distribution of expression values of *GAPDH* in the macrophage samples was stable and comparable between the low-HDL and control groups.

Carotid artery ultrasonography

B-mode ultrasound imaging was used to measure the intima-media complex of the carotid artery wall with a Hewlett-Packard (Andover, MA) Image Point M2410A ultrasound system as described previously (32). The average of all mean IMT measurements (mean IMT) over 28 sites (the far wall and the near wall of six arterial segments: right and left common carotid artery, carotid bulb, and proximal internal carotid artery) was chosen as the primary outcome variable. A single physician (A.H.) carried out all ultrasound examinations. To estimate the intrasonographer variability in the scannings, 10 subjects were scanned twice on two different occasions. The intraobserver repeatability for mean IMT was 0.971 with a SEM of 0.029.

Statistical methods

Statistical comparisons of clinical and biomedical parameters were performed with SPSS 11.0 for Windows. Differences in continuous variables between groups were analyzed by ANOVA. $P < 0.050$ (two-tailed *t*-test) was considered significant. Variables with skewed distribution were $\log_{10}$-transformed before the analyses. A Chi-square test with Fisher's exact test was used to determine significant differences in the allele frequencies and categorical variables between groups. Bivariate correlations between the continuous variables were calculated using the parametric Pearson correlation analysis. Adjustment for BMI and waist circumference was performed by treating these factors as covariates in the General Linear Model Univariate analysis and saving the nonstandardized residual value as the adjusted variable. Adjustment for statin use was performed by treating this as a fixed factor in the General Linear Model Univariate analysis. Multivariate stepwise linear regression analysis was conducted by removing the independent variables from the model until the best-fitting model with the maximum multiple R^2 was achieved.

RESULTS

Clinical characteristics of the study subjects

Clinical and biochemical characteristics of the study subjects are presented in Table 1 and supplementary Table I. Low-HDL subjects and controls had similar age distribution. By definition, HDL-C and apoA-I were decreased in the low-HDL group. They also had significantly lower total preβ-HDL mass and smaller mean HDL particle size than the controls. The low-HDL subjects had increased levels of TG and TC, and they had higher waist circumference and BMI than the controls. Hypertension was recorded in nine low-HDL subjects and five controls, and six low-HDL subjects were currently smokers compared with one in the control group. Mean IMT was increased significantly in the low-HDL group (1.02 ± 0.21 vs. 0.86 ± 0.17 mm; $P = 0.007$). This difference remained statistically significant

after adjustment for BMI ($P = 0.022$) and marginally significant after adjustment for waist circumference ($P = 0.053$). From the two lipid transfer proteins analyzed, significantly increased CETP activity was observed among the low-HDL subjects, whereas PLTP activity did not differ between the groups. PLTP mass was lower in low-HDL subjects. The prevalence of CHD was high within the low-HDL group (17 individuals), and 13 of them were on statin medication. The lipid values originally used for the phenotype determination in the recruitment phase were taken before lipid-lowering medication was started, but the values presented in the text and tables were acquired during medication. Because statins affect lipid status and can cause bias in the interpretation of the metabolic data, we divided the individuals within the low-HDL group into two subgroups based on their statin use. All 13 affected low-HDL subjects treated with statins had CHD, whereas among nonusers, 3 were CHD patients. Those who were treated with statins had similar cholesterol efflux (%) from macrophages compared with that among nonusers. As expected, TC was decreased significantly in the statin users. The two subgroups did not differ in age, HDL-C, apolipoprotein, or preβ-HDL levels. The *P* values for the between-group comparisons in Table 1 are presented before and after adjustment for statin use. None of the study subjects was diabetic.

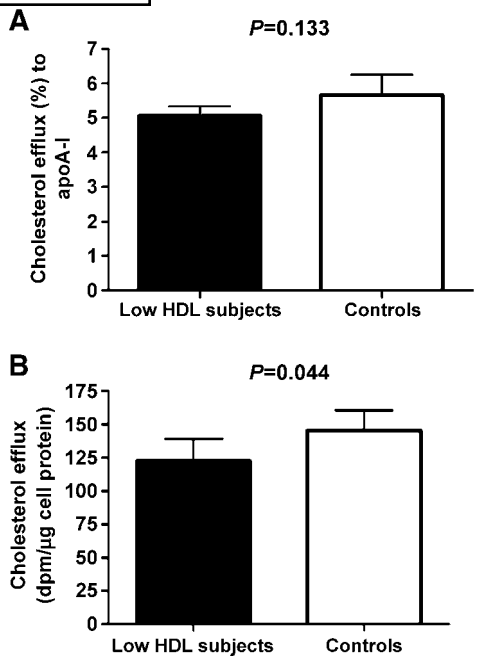

Fig. 1. Apolipoprotein A-I (apoA-I)-mediated cellular cholesterol efflux in low-HDL subjects (n = 22) and control subjects (n = 21). A: ABCA1-mediated cholesterol efflux to lipid-free apoA-I calculated as percentage efflux. B: ABCA1-mediated cholesterol efflux to lipid-free apoA-I normalized to macrophage protein content (dpm in medium/μg cell protein/18 h). Error bars represent mean ± SEM.

Macrophage cholesterol efflux to apoA-I and its relation to ABCA1 mRNA expression in cholesterol-loaded macrophages

We first investigated whether monocyte-derived macrophages of low-HDL subjects would display signs of dysfunction in the initial step of RCT. As shown in **Fig. 1A**, the low-HDL subjects (n = 22) displayed similar *ABCA1*-mediated cholesterol efflux to lipid-free apoA-I (calculated as percentage efflux) as the control group (n = 21) (5.1 ± 1.3 vs. 5.7 ± 1.3; $P = 0.133$). This result did not change after adjustment for statin therapy. There were no differences in cholesterol efflux levels in the low-HDL subjects with CHD (n = 16) compared with those without CHD (n = 6) (see supplementary Table II). In contrast, when cholesterol efflux was normalized to macrophage protein content (dpm in medium/μg cell protein/18 h), the rate of efflux was slightly slower in the low-HDL group compared with that in the control group (Fig. 1B). Importantly, the ability to become loaded with cholesteryl esters after incubation with acetyl-LDL did not differ between the macrophages derived from the low-HDL subjects and those derived from the control subjects (data not shown).

We next analyzed steady-state transcripts of *ABCA1* and *ABCG1* in the cultured monocyte-macrophages. Sufficient amounts of mRNA were available from only 10 low-HDL and 11 control subjects. Notably, the relative *ABCA1* expression in cholesterol-loaded macrophages was significantly higher in the low-HDL group than in the control group (**Fig. 2A**). The absolute *ABCA1* expression levels decayed after cholesterol efflux to apoA-I, and the differences between the groups disappeared. Unlike the transcript levels of *ABCA1*, the relative *ABCG1* transcript levels did not differ significantly between the groups either in macrophages after cholesterol loading or in macrophages after the efflux period (data not shown). In light of this clear difference in *ABCA1* transcript levels but no clear difference in percentage efflux to apoA-I, we next expressed efflux by cholesterol efflux to apoA-I (%)/relative *ABCA1* mRNA expression. This ratio was significantly reduced in the low-HDL group compared with the controls (3.93 ± 2.30 vs. 7.85 ± 2.14; $P < 0.001$) (Fig. 2B). This difference remained statistically significant after adjustment for BMI ($P = 0.004$) and waist circumference ($P = 0.016$) but not after adjustment for lipid medication. *ABCG1* expression in macrophages of low-HDL subjects followed the same trend as that of *ABCA1*, being highest in the cholesterol-loaded cells and declining during the efflux process (data not shown).

Next, we analyzed potential correlations between efflux (using cholesterol efflux percentage to apoA-I) and various parameters listed in Table 1. In the low-HDL group, cholesterol efflux showed a positive association with circulating preβ-HDL levels ($r = 0.504$, $P = 0.017$), whereas no correlation could be observed in the control group. Cholesterol efflux did not correlate significantly with mean IMT or HDL-C in the low-HDL group or in the control group (data not shown). The mean IMT was strongly positively correlated with age in both low-HDL subjects ($r = 0.709$, $P < 0.001$) and control subjects ($r = 0.746$, $P < 0.001$). In the multivariate linear regression model for mean IMT, the variables that entered the final model were

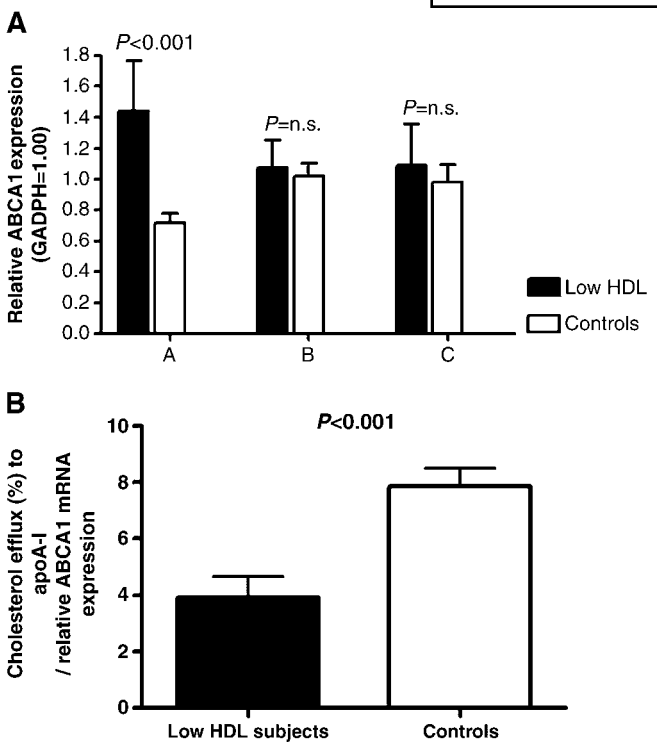

Fig. 2. *ABCA1* expression and cholesterol efflux relative to ABCA1 transcripts in macrophage foam cells among low-HDL and control subjects. A: Relative *ABCA1* mRNA expression in low-HDL subjects (n = 10) and control subjects (n = 11). Monocyte-derived macrophages were collected for mRNA extraction after cholesterol loading (A bars), after efflux in the absence of apoA-I (B bars), and after efflux in the presence of apoA-I (C bars). The bars present mean relative expression within the group ± 95% confidence interval normalized against the expression of GAPDH. The GAPDH expression is set at 1.00. B: Cholesterol efflux (percentage cholesterol efflux to apoA-I/relative *ABCA1* mRNA expression in cholesterol-loaded macrophages) in low-HDL subjects (n = 10) compared with control subjects (n = 11). Bars show means ± 95% confidence interval of the mean.

age, cholesterol efflux, and smoking, with an adjusted multiple R^2 of 0.613 (in low-HDL and control subjects pooled together; n = 43) (individual correlation coefficients are presented in **Table 2**).

Allelic diversity of the *ABCA1* gene and HDL levels

Motivated by the observed differences in *ABCA1* expression, we first addressed the allelic diversity of the subjects in the efflux experiment (n = 21) by genotyping 15 intragenic SNPs of the *ABCA1* gene. Analysis of the genotypes revealed an association of two SNPs with the ABCA1-mediated cholesterol efflux (rs2740492, $P = 0.030$; rs3858075, $P = 0.018$). The genotyping was subsequently extended to include the nonaffected family members (n = 72). Three SNPs differed significantly between the low-HDL family members and control subjects [L158 (rs2230805), $P = 0.004$; R219K (rs2230806), $P = 0.005$; and rs2297409, $P = 0.006$], so that the rare alleles of each of these SNPs were overrepresented among low-HDL subjects **(Table 3)**. The variants L158 and R219K showed significant association with low HDL-C levels ($P = 0.009$ and $P = 0.007$, respectively), and T1427 showed association with high HDL-C ($P = 0.028$). One SNP (L158) showed association with lower cholesterol efflux ($P = 0.009$), and one SNP (rs4149341) showed association with increased mean IMT ($P = 0.025$). We constructed allelic haplotypes of the three SNPs that showed association with either high or low HDL-C levels (L158, R219K, and T1427T) using PHASE version 2.0 software. Because of the linkage disequilibrium between these variants, only five different haplotypes could be identified in our study sample. The carrier status for the most common haplotype correlated with the HDL-C levels in a dose-dependent manner: the mean HDL-C level of individuals with two copies was 1.30 mmol/l, that for individuals with one copy was 1.06 mmol/l, and that for individuals with no copies was 0.85 mmol/l ($P = 0.025$, Kruskal-Wallis test). The likelihood of an individual belonging to the low-HDL group when carrying zero, one, or two copies of this allelic

TABLE 2. Multivariate regression analysis for mean IMT and predictor variables when low HDL subjects (n = 22) and control subjects (n = 21) were pooled together (n = 43)

Independent Variables	Standard Coefficient	SEM	*P*
Age	0.547	0.001	<0.001
Cigarette years	0.356	0.001	0.003
Cholesterol efflux (%)	−0.227	0.007	0.042
Adjusted multiple R^2	0.613		

Variables excluded from the model were preβ-HDL (%), apoA-I, apoA-II, HDL-C, BMI, TC, apoB, CETP activity, PLTP activity, systolic blood pressure, TG, homeostasis model assessment of insulin resistance (HOMA IR), HDL particle size, waist circumference, and statin use.

TABLE 3. Comparisons of the *ABCA1* allele frequencies between 47 low-HDL family members (19 unaffected and 28 affected subjects) and 25 control subjects, and *ABCA1* genotype effects on cholesterol efflux, HDL-C, and mean IMT in the pooled study group (n = 72)

Single-Nucleotide Polymorphism Identification Number	Amino Acid Residue	Allele Frequencies and *P* Value in Low-HDL Family Members Versus Control Subjects[a]	HDL-C P^b	Mean IMT P^c
rs2472459	—	NS	NS	NS
rs2246293	—	NS	NS	NS
rs2515616	—	NS	NS	NS
rs1800978	—	NS	NS	NS
rs2740492	—	NS	NS	NS
rs3858075	—	NS	NS	NS
rs1929842	—	NS	NS	NS
rs2230805	L158	(0.45 vs. 0.12) 0.004	0.009	NS
rs2230806	R219K	(0.40 vs. 0.08) 0.005	0.007	NS
rs2487037	—	NS	NS	NS
rs2297409	—	(0.30 vs. 0.04) 0.006	NS	NS
rs2066716	T1427	(0.11 vs. 0.32) 0.053	0.003	NS
rs2230808	R1587K	NS	NS	NS
rs2066881	—	NS	NS	NS
rs4149341	—	NS	NS	0.025

[a] For statistically significant differences, the corresponding allele frequencies are given in parentheses (in low-HDL family members vs. control subjects), followed by Chi-square *P* value, with Fisher's exact test for the differences in allele frequencies between low-HDL family members and control subjects.
[b] ANOVA *P* value for the *ABCA1* genotype effect on HDL-C.
[c] ANOVA *P* value for the *ABCA1* genotype effect on mean IMT.

haplotype was 80, 48, or 30%, respectively. The observed differences in the allele frequencies also remained statistically significant when tested in the study group used in functional analyses for whom cholesterol efflux to apoA-I was measured (22 low-HDL and 21 control subjects). Interestingly, the carrier status of the haplotype containing the R219K variant was not associated with the cholesterol efflux activity, implying that this particular variant does not result in the disturbed function of ABCA1 in macrophages but rather tags the allelic variant related to serum HDL-C level regulation, possibly at the hepatic level.

Interestingly, the carriers of the rare alleles in L158 had decreased efflux (%), like noncarriers in the whole study sample (4.6 ± 1.0 vs. 5.7 ± 1.3; $P = 0.009$). They also had lower HDL-C (1.00 ± 0.33 vs. 1.29 ± 0.46; $P = 0.011$). The relative ABCA1 expression was not significantly different between carriers and noncarriers. The efflux (%) was not changed significantly between carriers and noncarriers of the rare alleles in R219K. However, the carriers had lower HDL-C (0.99 ± 0.34 vs. 1.30 ± 0.46; $P = 0.008$) and increased relative ABCA1 expression (1.61 ± 0.31 vs. 1.02 ± 0.46; $P = 0.013$).

DISCUSSION

This study is the first comprehensive analysis of the macrophage cholesterol efflux efficiency in patients with familial low HDL-C, not representing Tangier patients. The *ABCA1*-dependent cholesterol efflux was measured in a foam cell model measuring *1*) cholesterol loading of macrophages isolated from the study subjects and *2*) unloading of macrophage-derived foam cells by incubation with lipid-free apoA-I as a primary cholesterol acceptor. We found that macrophages derived from low-HDL subjects expressed similar fractional cholesterol efflux (%) as those isolated from the control subjects. However, their cholesterol efflux estimated as percentage efflux (to apoA-I) relative to the levels of ABCA1 transcripts was reduced significantly. Although low-HDL subjects had markedly increased mean IMT, the arterial thickening was not associated with decreased cholesterol efflux. This finding might be related to the relatively small number of subjects participating in the cholesterol efflux study.

The efflux methodology that we applied to measure ABCA1-facilitated cholesterol efflux to apoA-I has been used widely previously (33, 34). Currently, different methods can be used to calculate the amount of cholesterol efflux from macrophage foam cells to acceptors. In this study, two of the most common approaches were used: *1*) cholesterol efflux to apoA-I expressed as fractional efflux (%), and *2*) cholesterol efflux to apoA-I expressed as the release of radioactivity to medium relative to cell protein (dpm/μg protein/18 h). Interestingly, although cholesterol efflux calculated as a percentage did not differ between the low-HDL subjects and the control group, there was a trend for reduced efflux ($P = 0.044$) among the low-HDL subjects when the efflux data were normalized to the cell protein. This discrepancy could be attributable to statistical limitations (i.e., comparison of two parameters with scale differences, and the relatively limited number of individuals per group attributable to the analytical complexity of the study). Next, we determined the abundance of *ABCA1* transcript after loading of the macrophages and demonstrated significantly higher expression levels in the foam cells derived from low-HDL subjects. To compare the efflux between the two groups, we expressed efflux (as a percentage or normalized for the cellular protein) relative to ABCA1 transcript and found that macrophage foam cells from the low-HDL subjects had reduced efflux of cholesterol compared with the control subjects. The following possibilities must be considered to explain this result. First, it is possible that the transcript levels do not correlate with the *ABCA1* protein abundance, because posttranscriptional regulation is an important determinant of *ABCA1* activity (35). Second, the *ABCA1* protein in the low-HDL subjects may be abnormally unstable; therefore, the observed increase in mRNA expression could reflect a compensatory mechanism caused by a faster degradation rate of the polypeptide. Finally, the observed higher transcript level of *ABCA1* may reflect a defective efflux in specific *ABCA1* variants in the macrophages of low-HDL patients. The relative mRNA expression levels of *ABCG1* in macrophages after either cholesterol loading or apoA-I-mediated efflux were not significantly different between low-HDL subjects and control subjects. However, as observed with *ABCA1*, the expression of *ABCG1* was highest in the macrophage foam cells and decreased significantly after cholesterol efflux, also reflecting the known regulatory effects of cellular cholesterol loading and unloading on both genes (36).

It has been shown that the most effective acceptor of ABCG1-derived cholesterol is the mature, spherical HDL, and not the lipid-poor apoA-I or preβ-HDL (20, 22). This finding may explain the early data indicating that individuals with high levels of large HDL_2 particles are protected from atherosclerosis (37, 38). In mice with a targeted disruption of *ABCG1*, the absence of functional protein causes an accumulation of neutral lipids and phospholipids in macrophages and hepatocytes (39). The human *ABCG1* gene is located on chromosome 21q22.3 (40), and to date, eight transcriptional variants have been characterized. However, no human disorders attributable to either heterozygous or homozygous defects in the *ABCG1* gene have been described. In turn, some data exist that ABCG1 is overexpressed in macrophages from Tangier patients compared with control macrophages (41).

When addressing the allelic diversity of the *ABCA1* gene, we observed that low-HDL subjects carried rare *ABCA1* alleles significantly more often than control subjects. Four SNPs differed in allele frequency between the low-HDL and control groups. A previous study in a separate sample of Finnish families with premature CHD and low HDL-C reported no effect of the *ABCA1* locus on HDL-C levels (16). Negative findings for the common *ABCA1* variants were also reported in the Veterans Affairs HDL Intervention Trial and the Framingham Offspring Study (15). In turn, in healthy subjects and CHD patients of European

and French-Canadian origin, the common variant R219K was associated with increased levels of HDL, decreased TGs, and reduced severity of atherosclerosis (42, 43). In a recent elegant approach, a systematic screening of *ABCA1* in a large Danish population sample revealed that of the subjects in the lowest percentile of HDL-C levels, 10% are heterozygous for *ABCA1* mutations and that common *ABCA1* SNPs in fact do contribute to HDL-C concentrations at the population level (14). In this study, we genotyped five of the SNPs reported in the Danish population, of which three were associated with HDL-C levels (ANOVA $P < 0.050$). Of these, T1427 gave corresponding results in both data sets, showing association with increased HDL-C. The variant R219K showed association with low serum HDL levels but not with the cholesterol efflux in our study sample. Interestingly, the L158 polymorphism (rs2230805) did correlate with percentage efflux; however, being a synonymous SNP, it is in itself unlikely to be functional. Although the common *ABCA1* haplotype did not associate with cholesterol efflux but did relate to serum HDL levels, it could be important for the liver-specific processing of ABCA1, thus explaining why it did not correlate with the level of macrophage cholesterol efflux.

A number of study limitations should be considered. First, the limited number of subjects available for the cholesterol efflux experiments may attenuate the statistical power. The variable yield of cells (monocytes) isolated for the efflux experiments and *ABCA1* mRNA evaluation is another possible flaw. Complete sequencing of the *ABCA1* gene would possibly identify carriers of rare variants in a larger study sample, but as previous sequencing in these Finnish low-HDL families failed to find any novel, rare variants, we are confident that our haplotype analysis based on 15 tagging SNPs exposes the detectable allelic diversity in our samples. Finally, lack of simultaneous data on the actual ABCA1/G1 protein level is a weakness. Specifically, in addition to defining transcript levels of ABC transporters, additional studies are necessary to determine the correlation between cholesterol efflux and the levels of ABCA1/G1 proteins in low-HDL subjects. Because accurate quantification of ABC transporter protein levels cannot be achieved with semiquantitative Western blot analyses, the development of an ELISA-type immunoassay would be necessary for such purposes.

In summary, based on our data, we conclude that *1*) macrophages derived from Finnish subjects with familial low HDL displayed similar cholesterol-loading capacity but decreased cholesterol efflux to apoA-I via the ABCA1 pathway after loading; *2*) familial low HDL in these families is not caused by rare mutations in *ABCA1*; rather, the common allelic variants are associated with HDL-C levels, as indicated by the dose-dependent contribution of the haplotype carrying the common R219K change in these subjects; and *3*) relative *ABCA1* mRNA expression in cholesterol-loaded macrophages was increased significantly in the low-HDL group. We propose that defective ABCA1 function in cholesterol-loaded macrophages is one potential contributor to the impaired RCT process in low-HDL subjects.

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REFERENCES

1. Stein, O., and Y. Stein. 1999. Atheroprotective mechanisms of HDL. *Atherosclerosis.* **144:** 285–301.
2. Bodzioch, M., E. Orso, J. Klucken, T. Langmann, A. Bottcher, W. Diederich, W. Drobnik, S. Barlage, C. Buchler, M. Porsch-Ozcurumez, et al. 1999. The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease. *Nat. Genet.* **22:** 347–351.
3. Brooks-Wilson, A., M. Marcil, S. M. Clee, L. H. Zhang, K. Roomp, M. van Dam, L. Yu, C. Brewer, J. A. Collins, H. O. Molhuizen, et al. 1999. Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency. *Nat. Genet.* **22:** 336–345.
4. Rust, S., M. Rosier, H. Funke, J. Real, Z. Amoura, J. Piette, J. Deleuze, H. Brewer, N. Duverger, P. Denefle, et al. 1999. Tangier disease is caused by mutations in the gene encoding ATP-binding cassette transporter 1. *Nat. Genet.* **22:** 352–355.
5. Brewer, H. B., Jr., A. T. Remaley, E. B. Neufeld, F. Basso, and C. Joyce. 2004. Regulation of plasma high-density lipoprotein levels by the ABCA1 transporter and the emerging role of high-density lipoprotein in the treatment of cardiovascular disease. *Arterioscler. Thromb. Vasc. Biol.* **24:** 1755–1760.
6. Timmins, J. M., J. Y. Lee, E. Boudyguina, K. D. Kluckman, L. R. Brunham, A. Mulya, A. K. Gebre, J. M. Coutinho, P. L. Colvin, T. L. Smith, et al. 2005. Targeted inactivation of hepatic Abca1 causes profound hypoalphalipoproteinemia and kidney hypercatabolism of apoA-I. *J. Clin. Invest.* **115:** 1333–1342.
7. Brunham, L. R., J. K. Kruit, J. Iqbal, C. Fievet, J. M. Timmins, T. D. Pape, B. A. Coburn, N. Bissada, B. Staels, A. K. Groen, et al. 2006. Intestinal ABCA1 directly contributes to HDL biogenesis in vivo. *J. Clin. Invest.* **116:** 1052–1062.
8. Haghpassand, M., P. A. Bourassa, O. L. Francone, and R. J. Aiello. 2001. Monocyte/macrophage expression of ABCA1 has minimal contribution to plasma HDL levels. *J. Clin. Invest.* **108:** 1315–1320.
9. Clee, S. M., J. J. Kastelein, M. van Dam, M. Marcil, K. Roomp, K. Y. Zwarts, J. A. Collins, R. Roelants, N. Tamasawa, T. Stulc, et al. 2000. Age and residual cholesterol efflux affect HDL cholesterol levels and coronary artery disease in ABCA1 heterozygotes. *J. Clin. Invest.* **106:** 1263–1270.
10. Brousseau, M. E., G. P. Eberhart, J. Dupuis, B. F. Asztalos, A. L. Goldkamp, E. J. Schaefer, and M. W. Freeman. 2000. Cellular cholesterol efflux in heterozygotes for Tangier disease is markedly reduced and correlates with high density lipoprotein cholesterol concentration and particle size. *J. Lipid Res.* **41:** 1125–1135.
11. van Dam, M. J., E. de Groot, S. M. Clee, G. K. Hovingh, R. Roelants, A. Brooks-Wilson, A. H. Zwinderman, A. J. Smit, A. H. Smelt, A. K. Groen, et al. 2002. Association between increased arterial-wall thickness and impairment in ABCA1-driven cholesterol efflux: an observational study. *Lancet.* **359:** 37–42.
12. Wang, J., J. R. Burnett, S. Near, K. Young, B. Zinman, A. J. Hanley, P. W. Connelly, S. B. Harris, and R. A. Hegele. 2000. Common and rare ABCA1 variants affecting plasma HDL cholesterol. *Arterioscler. Thromb. Vasc. Biol.* **20:** 1983–1989.
13. Hong, S. H., J. Rhyne, K. Zeller, and M. Miller. 2002. ABCA1 (Alabama): a novel variant associated with HDL deficiency and premature coronary artery disease. *Atherosclerosis.* **164:** 245–250.
14. Frikke-Schmidt, R., B. G. Nordestgaard, G. B. Jensen, and A.

Tybjaerg-Hansen. 2004. Genetic variation in ABC transporter A1 contributes to HDL cholesterol in the general population. *J. Clin. Invest.* **114:** 1343–1353.
15. Brousseau, M. E., M. Bodzioch, E. J. Schaefer, A. L. Goldkamp, D. Kielar, M. Probst, J. M. Ordovas, C. Aslanidis, K. J. Lackner, H. Bloomfield Rubins, et al. 2001. Common variants in the gene encoding ATP-binding cassette transporter 1 in men with low HDL cholesterol levels and coronary heart disease. *Atherosclerosis.* **154:** 607–611.
16. Kakko, S., J. Kelloniemi, P. von Rohr, I. Hoeschele, M. Tamminen, M. E. Brousseau, Y. A. Kesaniemi, and M. J. Savolainen. 2003. ATP-binding cassette transporter A1 locus is not a major determinant of HDL-C levels in a population at high risk for coronary heart disease. *Atherosclerosis.* **166:** 285–290.
17. Soro, A., P. Pajukanta, H. E. Lilja, K. Ylitalo, T. Hiekkalinna, M. Perola, R. M. Cantor, J. S. Viikari, M. R. Taskinen, and L. Peltonen. 2002. Genome scans provide evidence for low-HDL-C loci on chromosomes 8q23, 16q24.1-24.2, and 20q13.11 in Finnish families. *Am. J. Hum. Genet.* **70:** 1333–1340.
18. Chen, H., C. Rossier, M. D. Lalioti, A. Lynn, A. Chakravarti, G. Perrin, and S. E. Antonarakis. 1996. Cloning of the cDNA for a human homologue of the Drosophila white gene and mapping to chromosome 21q22.3. *Am. J. Hum. Genet.* **59:** 66–75.
19. Klucken, J., C. Buchler, E. Orso, W. E. Kaminski, M. Porsch-Ozcurumez, G. Liebisch, M. Kapinsky, W. Diederich, W. Drobnik, M. Dean, et al. 2000. ABCG1 (ABC8), the human homolog of the Drosophila white gene, is a regulator of macrophage cholesterol and phospholipid transport. *Proc. Natl. Acad. Sci. USA.* **97:** 817–822.
20. Wang, N., D. Lan, W. Chen, F. Matsuura, and A. R. Tall. 2004. ATP-binding cassette transporters G1 and G4 mediate cellular cholesterol efflux to high-density lipoproteins. *Proc. Natl. Acad. Sci. USA.* **101:** 9774–9779.
21. Nakamura, K., M. A. Kennedy, A. Baldan, D. D. Bojanic, K. Lyons, and P. A. Edwards. 2004. Expression and regulation of multiple murine ATP-binding cassette transporter G1 mRNAs/isoforms that stimulate cellular cholesterol efflux to high density lipoprotein. *J. Biol. Chem.* **279:** 45980–45989.
22. Vaughan, A. M., and J. F. Oram. 2005. ABCG1 redistributes cell cholesterol to domains removable by high density lipoprotein but not by lipid-depleted apolipoproteins. *J. Biol. Chem.* **280:** 30150–30157.
23. Soro, A., M. Jauhiainen, C. Ehnholm, and M. R. Taskinen. 2003. Determinants of low HDL levels in familial combined hyperlipidemia. *J. Lipid Res.* **44:** 1536–1544.
24. van Haperen, R., A. van Tol, P. Vermeulen, M. Jauhiainen, T. van Gent, P. van den Berg, S. Ehnholm, F. Grosveld, A. van der Kamp, and R. de Crom. 2000. Human plasma phospholipid transfer protein increases the antiatherogenic potential of high density lipoproteins in transgenic mice. *Arterioscler. Thromb. Vasc. Biol.* **20:** 1082–1088.
25. Soderlund, S., A. Soro-Paavonen, C. Ehnholm, M. Jauhiainen, and M. R. Taskinen. 2005. Hypertriglyceridemia is associated with preβ-HDL concentrations in subjects with familial low HDL. *J. Lipid Res.* **46:** 1643–1651.
26. Watanabe, H., S. Soderlund, A. Soro-Paavonen, A. Hiukka, E. Leinonen, C. Alagona, R. Salonen, T. P. Tuomainen, C. Ehnholm, M. Jauhiainen, et al. 2006. Decreased high-density lipoprotein (HDL) particle size, pre{beta}-, and large HDL subspecies concentration in Finnish low-HDL families. Relationship with intima-media thickness. *Arterioscler. Thromb. Vasc. Biol.* **26:** 897–902.
27. Siggins, S., M. Karkkainen, J. Tenhunen, J. Metso, E. Tahvanainen, V. M. Olkkonen, M. Jauhiainen, and C. Ehnholm. 2004. Quantitation of the active and low-active forms of human plasma phospholipid transfer protein by ELISA. *J. Lipid Res.* **45:** 387–395.
28. Pastinen, T., M. Raitio, K. Lindroos, P. Tainola, L. Peltonen, and A. C. Syvanen. 2000. A system for specific, high-throughput genotyping by allele-specific primer extension on microarrays. *Genome Res.* **10:** 1031–1042.
29. Rothblat, G. H., M. de la Llera-Moya, V. Atger, G. Kellner-Weibel, D. L. Williams, and M. C. Phillips. 1999. Cell cholesterol efflux: integration of old and new observations provides new insights. *J. Lipid Res.* **40:** 781–796.
30. Lindstedt, L., M. Lee, G. R. Castro, J. C. Fruchart, and P. T. Kovanen. 1996. Chymase in exocytosed rat mast cell granules effectively proteolyzes apolipoprotein AI-containing lipoproteins, so reducing the cholesterol efflux-inducing ability of serum and aortic intimal fluid. *J. Clin. Invest.* **97:** 2174–2182.
31. Pajukanta, P., H. E. Lilja, J. S. Sinsheimer, R. M. Cantor, A. J. Lusis, M. Gentile, X. J. Duan, A. Soro-Paavonen, J. Naukkarinen, J. Saarela, et al. 2004. Familial combined hyperlipidemia is associated with upstream transcription factor 1 (USF1). *Nat. Genet.* **36:** 371–376.
32. Ylitalo, K., M. Syvanne, R. Salonen, I. Nuotio, M. R. Taskinen, and J. T. Salonen. 2002. Carotid artery intima-media thickness in Finnish families with familial combined hyperlipidemia. *Atherosclerosis.* **162:** 171–178.
33. Chinetti, G., S. Lestavel, V. Bocher, A. T. Remaley, B. Neve, I. P. Torra, E. Teissier, A. Minnich, M. Jaye, N. Duverger, et al. 2001. PPAR-alpha and PPAR-gamma activators induce cholesterol removal from human macrophage foam cells through stimulation of the ABCA1 pathway. *Nat. Med.* **7:** 53–58.
34. Rigamonti, E., L. Helin, S. Lestavel, A. L. Mutka, M. Lepore, C. Fontaine, M. A. Bouhlel, S. Bultel, J. C. Fruchart, E. Ikonen, et al. 2005. Liver X receptor activation controls intracellular cholesterol trafficking and esterification in human macrophages. *Circ. Res.* **97:** 682–689.
35. Yokoyama, S. 2005. Assembly of high density lipoprotein by the ABCA1/apolipoprotein pathway. *Curr. Opin. Lipidol.* **16:** 269–279.
36. Schmitz, G., and T. Langmann. 2005. Transcriptional regulatory networks in lipid metabolism control ABCA1 expression. *Biochim. Biophys. Acta.* **1735:** 1–19.
37. Freedman, D. S., J. D. Otvos, E. J. Jeyarajah, J. J. Barboriak, A. J. Anderson, and J. A. Walker. 1998. Relation of lipoprotein subclasses as measured by proton nuclear magnetic resonance spectroscopy to coronary artery disease. *Arterioscler. Thromb. Vasc. Biol.* **18:** 1046–1053.
38. Asztalos, B. F., P. S. Roheim, R. L. Milani, M. Lefevre, J. R. McNamara, K. V. Horvath, and E. J. Schaefer. 2000. Distribution of apoA-I-containing HDL subpopulations in patients with coronary heart disease. *Arterioscler. Thromb. Vasc. Biol.* **20:** 2670–2676.
39. Kennedy, M. A., G. C. Barrera, K. Nakamura, A. Baldan, P. Tarr, M. C. Fishbein, J. Frank, O. L. Francone, and P. A. Edwards. 2005. ABCG1 has a critical role in mediating cholesterol efflux to HDL and preventing cellular lipid accumulation. *Cell Metab.* **1:** 121–131.
40. Kennedy, M. A., A. Venkateswaran, P. T. Tarr, I. Xenarios, J. Kudoh, N. Shimizu, and P. A. Edwards. 2001. Characterization of the human ABCG1 gene: liver X receptor activates an internal promoter that produces a novel transcript encoding an alternative form of the protein. *J. Biol. Chem.* **276:** 39438–39447.
41. Lorkowski, S., M. Kratz, C. Wenner, R. Schmidt, B. Weitkamp, M. Fobker, J. Reinhardt, J. Rauterberg, E. A. Galinski, and P. Cullen. 2001. Expression of the ATP-binding cassette transporter gene ABCG1 (ABC8) in Tangier disease. *Biochem. Biophys. Res. Commun.* **283:** 821–830.
42. Clee, S. M., A. H. Zwinderman, J. C. Engert, K. Y. Zwarts, H. O. Molhuizen, K. Roomp, J. W. Jukema, M. van Wijland, M. van Dam, T. J. Hudson, et al. 2001. Common genetic variation in ABCA1 is associated with altered lipoprotein levels and a modified risk for coronary artery disease. *Circulation.* **103:** 1198–1205.
43. Tregouet, D. A., S. Ricard, V. Nicaud, I. Arnould, S. Soubigou, M. Rosier, N. Duverger, O. Poirier, S. Mace, F. Kee, et al. 2004. In-depth haplotype analysis of ABCA1 gene polymorphisms in relation to plasma apoA1 levels and myocardial infarction. *Arterioscler. Thromb. Vasc. Biol.* **24:** 775–781.