Cholesteryl ester transfer protein and hyperalphalipoproteinemia in Caucasians

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Abstract It is unclear whether cholesteryl ester transfer protein (CETP) contributes to high density lipoprotein cholesterol (HDL-C) levels in hyperalphalipoproteinemia (HALP) in Caucasians. Moreover, even less is known about the effects of hereditary CETP deficiency in non-Japanese. We studied 95 unrelated Caucasian individuals with HALP. No correlations between CETP concentration or activity and HDL-C were identified. Screening for CETP gene defects led to the identification of heterozygosity for a novel splice site mutation in one individual. Twenty-five heterozygotes for this mutation showed reduced CETP concentration (−40%) and activity (−50%) and a 35% increase of HDL-C compared with family controls. The heterozygotes presented with an isolated high HDL-C, whereas the remaining subjects exhibited a typical high HDL-C/low-triglyceride phenotype. The increase of HDL-C in the CETP-deficient heterozygotes was primarily attributable to increased high density lipoprotein containing apolipoprotein AI and A-II (LpAI:AII) levels, contrasting with an increase in both high density lipoprotein containing apolipoprotein A-I only and LpAI:AII in the other group. This study suggests the absence of a relationship between CETP and HDL-C levels in Caucasians with HALP. The data furthermore indicate that genetic CETP deficiency is rare among Caucasians and that this disorder presents with a phenotype that is different from that of subjects with HALP who have no mutation in the CETP gene.—van der Steeg, W. A., G. K. Hovingh, A. H. E. M. Klerkx, B. A. Hutten, I. C. Nootenboom, J. H. M. Levels, A. van Tol, G. M. Dallinga-Thie, A. H. Zwinderman, J. J. P. Kastelein, and J. A. Kuivenhoven.

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Supplementary key words deficiency • mutation • CETP-IVS7+1

In the general population, variations of high density lipoprotein cholesterol (HDL-C) levels are commonly ascribed to a combination of environmental and genetic factors (1). By contrast, extremely high levels of HDL-C [hyperalphalipoproteinemia (HALP)] are often the result of monogenetic disturbances. Among the genetic factors identified to date [such as scavenger receptor class B type 1 (2, 3) and apolipoprotein C-III (apoC-III) deficiency (4)], deficiency of cholesteryl ester transfer protein (CETP) is a well-established cause of HALP (5). The CETP gene (6) is located on the long arm of chromosome 16 (7) and comprises 16 exons (8). In the circulation, CETP mediates the transfer of cholesteryl esters (CEs) from HDL to apoB-containing lipoproteins (VLDL, intermediate density lipoprotein, LDL) (9, 10). Accordingly, human CETP deficiency is characterized by CE enrichment of HDL, which results in increased levels of HDL-C. This observation has led to the development of small molecular inhibitors of CETP that have been shown to increase plasma levels of HDL-C in humans (11–14).

The central role of CETP in human HDL metabolism only became evident after the identification of Japanese subjects with genetic CETP deficiency. Homozygotes for loss-of-function mutations showed up to four times increased HDL-C levels (15, 16). Since this discovery in 1989, CETP mutations have been identified primarily in Japan, and Maruyama et al. (17) showed that genetic CETP deficiency is in fact a very frequent cause of HALP in that country. Heterozygosity for a splice donor acceptor site mutation in intron 14 (Int14+1 G→A) (15) and heterozygosity for a missense mutation in exon 15 (D442G) (16) were reported to underlie 74% and 62% increased

Abbreviations: apoC-III, apolipoprotein C-III; BMI, body mass index; CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; HALP, hyperalphalipoproteinemia; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; LpAI, high density lipoprotein containing apolipoprotein A-I only; LpAI:AII, high density lipoprotein containing apolipoprotein A-I and A-II.

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HDL-C levels compared with controls, respectively. Loss of CETP activity was further characterized by increased HDL\textsubscript{2} cholesterol concentration (18) and increased mean HDL particle size (19). Given the central role of CETP in exchanging neutral lipids between HDL and apoB-containing lipoproteins, it is expected that CETP deficiency would also affect (V)LDL metabolism. However, heterozygotes for Int14+1 G→A or D442G have not been reported to exhibit changes in low density lipoprotein cholesterol (LDL-C), apoB, or triglyceride levels (5), despite an \sim50\% reduction of CETP activity levels. Regarding LDL particle size, it has been shown that the dimensions of these particles are distributed over an atypically wide range in subjects with homozygous CETP deficiency (20), but data covering this specific issue are scarce.

Together, genetic CETP deficiency and the use of CETP inhibitors have shown that CETP has a strong impact on HDL metabolism and that loss of CETP activity generates favorable changes of lipid profiles. Data regarding genetic CETP deficiency, however, are derived mainly from subjects living in Japan. In Caucasians, the literature on CETP and HALP is scarce. In fact, only a few Caucasian individuals with CETP deficiency have been described in case reports (21–24), and it is not known whether CETP plays a major role in determining HDL-C levels in non-Japanese subjects. Therefore, the first objective of this study was to investigate the relationship between CETP and HDL-C levels in Caucasian subjects with HALP. The second objective was to screen for genetic CETP deficiency in this cohort. The identification of a large family with a novel CETP gene defect allowed us to study in depth the impact of this mutation on lipid metabolism.

**METHODS**

**Definition of the study group**

During the past decade, we have used our lipid clinic network and contacts with general practitioners in The Netherlands to collect plasma and DNA from individuals with HALP, with the intent to identify novel genes that control HDL-C levels. The vast majority of these individuals came to the attention of their physicians through general lipid tests that revealed high HDL-C levels. For this study, we examined 95 unrelated Caucasian index subjects (44 males, 51 females) from families in which HALP was established in at least three first-degree relatives. The average number of family members per index subject recruited was 14 and ranged from 4 to 342. Subjects were defined to have HALP if they presented with HDL-C levels above the 90th percentile for age and gender (for HDL-C percentile scale, see supplementary Table 1) at two visits, without the presence of secondary causes that could lead to a HALP phenotype (extensive regular aerobic exercise, regular intake of alcohol, estrogen replacement therapy, or drugs such as fibrates, nicotinic acid, and phenytoin). Nine of the 95 subjects received statin therapy for suspected (n = 1) or proven (n = 4) cardiovascular disease or for unknown reasons (n = 4). One of these subjects was additionally treated with a fibrate (gemfibrozil), but the presence of a HALP phenotype before the start of this therapy as well as the presence of this phenotype in three first-degree relatives were reasons for the inclusion of this individual.

Informed consent was obtained for blood sampling and genetic analyses, and the study was approved by the Medial Ethics Committee of the Academic Medical Center in Amsterdam, The Netherlands.

**Genomic CETP DNA sequence analysis and mutation screening**

To screen for genetic CETP deficiency, subjects with CETP concentrations < 1.4 \(\mu\)g/ml (which is the lower limit of the normal range for CETP concentration of our ELISA) were selected from the HALP group. CETP gene sequence analysis was subsequently performed when low CETP concentrations in these subjects cosegregated with the HALP phenotype (defined above) in at least one family member. Genomic DNA was isolated from peripheral blood leukocytes. All 16 exons of the CETP gene, including intron-exon boundaries (minimum of 50 nucleotides into intronic DNA), and 1,500 bp of the CETP promoter were amplified by standard PCR using CETP-specific primers based on GenBank sequence NT_024766. Sequence reactions were performed using the Big Dye Terminator ABI Prism kit on an Applied Biosystems model 310 automated DNA sequencer (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands).

Screening for the CETP-I14S+1 mutation was performed by restriction fragment length polymorphism analysis using the forward 5'-CCGTGCTTGTACACTAGT-3' and reverse 5'-CATCTCTGGTTGTCTT-3' primers for PCR and digestion with XcmI (New England Biolabs, Beverly, MA), which digests wild-type DNA but not the mutant sequence.

**RNA isolation and RT-PCR**

Total RNA was isolated from peripheral leukocytes. One volume of whole blood was mixed with 9 volumes of lysis buffer (155 mM NH\textsubscript{4}Cl, 10 mM KHCO\textsubscript{3}, and 0.1 mM EDTA) and kept on ice for 15 min. Cells were collected by centrifugation at 3,000 \(g\) and washed twice by resuspension in lysis buffer and repeated centrifugation. The pellet was resuspended in TrisPure, and RNA was isolated according to the manufacturer’s instructions (Roche Diagnostics Nederland BV, Almere, The Netherlands). First-strand cDNA was generated from 1 \(\mu\)g of total RNA with Superscript II Rnase \(H^-\) (Invitrogen, Breda, The Netherlands) with an oligo(dT) 14VN primer. Part of the cDNA reaction was used as template for a standard PCR. Primers used to detect alternative splicing products were 5'-TGGATCAAGCAGCTGTTCACA-3' and 5'-TGATGGGGACTCCAGGTAGGA-3', sequences derived from exons 6 and 8, respectively. PCR products were visualized on ethidium bromide-containing agarose gels and, if needed, excised and prepared for sequencing.

To quantify alternatively spliced CETP mRNA, mRNA was isolated using Magnapure isolation (Roche Diagnostics Nederland BV) from abdominal adipose tissue obtained by needle aspiration from a heterozygote for CETP-I14S+1 and two control subjects. The amount of total CETP mRNA (wild type and mutant) and wild-type CETP mRNA was determined with LightCycler SYBR Green quantification using primer sets in exons 2 and 3 and exons 6 and 7, respectively. GAPDH mRNA levels were determined as an internal control.

**Lipids, lipoproteins, apolipoproteins, and lipid-modifying proteins**

All measurements were performed on fasting blood samples. Lipids were measured using standardized techniques. CETP concentration was determined by ELISA (25), and plasma apoA-I, apoA-II, apoB, and apoE were measured by nephelometry (Dade Behring, Marburg, Germany). High density lipoprotein containing apolipoprotein A-I only (LpA1) and high density lipoprotein containing apolipoprotein A-I and A-II (LpA1A2) were measured with the nonspecific ELISA method.

**Genetic CETP mutation**

Information was obtained from the study of 95 unrelated Caucasian index subjects from families in which HALP was established in at least three first-degree relatives. The average number of family members per index subject recruited was 14 and ranged from 4 to 342.

**RNA sequence analysis**

Screening for CETP-I14S+1 was performed by restriction fragment length polymorphism analysis using the forward 5'-CCGTGCTTGTACACTAGT-3' and reverse 5'-CATCTCTGGTTGTCTT-3' primers for PCR and digestion with XcmI.

**Alternative splicing products**

Primers used to detect alternative splicing products were 5'-TGGATCAAGCAGCTGTTCACA-3' and 5'-TGATGGGGACTCCAGGTAGGA-3', sequences derived from exons 6 and 8, respectively.

**CETP mRNA levels**

CETP mRNA levels were determined using LightCycler SYBR Green quantification using primer sets in exons 2 and 3, exons 6 and 7, respectively. GAPDH mRNA levels were determined as an internal control.

**Lipid analysis**

All measurements were performed on fasting blood samples. Lipids were measured using standardized techniques. CETP concentration was determined by ELISA (25), and plasma apoA-I, apoA-II, apoB, and apoE were measured by nephelometry (Dade Behring, Marburg, Germany). High density lipoprotein containing apolipoprotein A-I only (LpA1) and high density lipoprotein containing apolipoprotein A-I and A-II (LpA1A2) were measured with the nonspecific ELISA method.
containing apolipoprotein A-I and A-II (LpA1AI) concentrations were determined by rocket gel electrophoresis (Sebia). Plasma CETP, LCAT, and phospholipid transfer protein activities were all measured using excess exogenous substrate methods (26). Activities are expressed as a percentage of the activity measured in pooled plasma obtained from 100 normolipidemic subjects. Lipoprotein subfraction concentrations and lipoprotein particle size were quantified by NMR spectrometry (27).

Statistical analysis
Analyses were performed using the Statistical Program for the Social Sciences (version 12.0.2; SPSS, Inc., Chicago, IL). P < 0.05 was considered significant. Continuous variables with a skewed distribution were log-transformed before analysis.

Pearson correlation coefficients (r) were calculated to examine the relationship between CETP concentrations, CETP activity, specific CETP activity, and HDL-C. Linear regression analysis was performed to examine these relationships adjusted for age, sex, body mass index (BMI), smoking, and statin/fibrate use when these factors contributed significantly to the model. Results of the linear regression analyses are presented as standardized coefficients (β).

A general linear model was used to compare lipids, (apo)lipoproteins, lipoprotein-modifying enzymes, and NMR data of the groups presented in Tables 1, 3, and 4. Using backward stepwise linear regression analysis, we again adjusted for age, sex, BMI, smoking, and statin/fibrate use when these parameters contributed significantly to the model. To investigate the effect of fibrate therapy, analyses were also performed after exclusion of the individual receiving fibrate therapy.

RESULTS

CETP and HALP
The first objective of this study was to investigate the relationship between CETP and HDL-C levels in Caucasian subjects with HALP. To this purpose, we studied 95 unrelated probands from families in which HALP was established in at least three first-degree relatives. The average HDL-C level in this group was 2.35 ± 0.42 mmol/l (Table 1), with higher HDL-C levels in women compared with men (2.61 ± 0.32 vs. 2.06 ± 0.33 mmol/l; P < 0.0001) (Table 1). Plasma CETP concentration was normally distributed and averaged 1.89 ± 0.60 μg/ml. Males presented with nonsignificantly lower CETP concentrations compared with females (1.79 ± 0.54 vs. 1.98 ± 0.60 μg/ml; P = 0.21) (Table 1). Mean plasma CETP activity in the entire group was similar to pooled plasma of healthy volunteers (99.0% vs. 100%). Mean CETP activity was slightly lower in men (96.8 ± 19.4%) compared with women (100.9 ± 29.7%), but this difference was not statistically significant (P = 0.42). Thus, compared with men, the women presented with higher HDL-C levels accompanied by nonsignificant increases in CETP concentration and activity (exclusion of the individual receiving fibrate therapy generated similar results).

Using the data for the entire group, we identified a correlation between CETP concentration and CETP activity (r = 0.67, P < 0.0001) (Table 2). Addressing the first question of this study, HDL-C levels were not correlated with CETP activity (r = 0.17, P = 0.16), CETP concentration (r = 0.14, P = 0.17), or specific activities of CETP (r = −0.17, P = 0.16). Linear regression analysis, adjusted for age, sex, BMI, smoking, and statin/fibrate use when necessary, generated similar results. CETP activity was assessed in only 71 of 95 subjects, as a result of lack of sufficient plasma.

Identification of a novel splice site mutation in the CETP gene in one family
Our second objective was to screen for genetic CETP deficiency in our cohort. To this purpose, we selected 13 subjects with CETP concentrations < 1.4 μg/ml (which is the lower limit of the normal range for CETP concentration of our ELISA). CETP gene sequence analysis was performed when low CETP concentrations in these subjects cosegregated with the HALP phenotype in at least one family member [this was the case in 12 (8 men, 4 women) of the 13 individuals with low CETP concentrations]. This analysis revealed a number of CETP gene variations (listed in supplementary Table II) that were either previously described polymorphisms (28, 29) or were not expected to affect CETP gene transcription, based on their positions in the CETP coding sequence.
Table 2. Relationships between CETP activity, CETP concentration, specific CETP activity, and HDL-C in 95 subjects with HDL-C levels above the 90th percentile for age and sex

<table>
<thead>
<tr>
<th>Variable</th>
<th>( r )</th>
<th>( P1 )</th>
<th>( \beta )</th>
<th>( P2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>CETP activity and CETP concentration</td>
<td>0.67</td>
<td>&lt;0.0001</td>
<td>0.67</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CETP activity and HDL-C</td>
<td>0.17</td>
<td>0.16</td>
<td>0.05</td>
<td>0.66</td>
</tr>
<tr>
<td>CETP concentration and HDL-C</td>
<td>0.14</td>
<td>0.17</td>
<td>0.11</td>
<td>0.30</td>
</tr>
<tr>
<td>Specific CETP activity and HDL-C</td>
<td>−0.17</td>
<td>0.16</td>
<td>−0.17</td>
<td>0.16</td>
</tr>
</tbody>
</table>

* \( r \): Pearson correlation coefficient; P1, level of significance; \( \beta \): standardized coefficient derived from linear regression analysis adjusting for age, sex, smoking, BMI, and statin/fibrate use when these parameters contributed significantly to the model; P2, level of significance.

* Assessed in 71 of 95 subjects (males, 33 of 44; females, 38 of 51) without selection bias.

Nonregulatory promoters or intronic sequences. We did not analyze these variations further, but focused on one woman who turned out to be heterozygous for a novel splice site mutation. It concerned a G→T nucleotide substitution at nucleotide +1 of intron 7 (CETP-IVS7+1). This sequence variation was not present in the remaining 94 probands with high HDL-C or in 200 normolipidemic unrelated control subjects.

We recruited 190 family members of this CETP-IVS7+1 proband and screened for this defect by PCR (see Methods for details). This led to the identification of 24 additional heterozygotes, but no homozygotes were found. Supplementary Fig. I shows the most relevant part of the pedigree. This led to the identification of 24 additional heterozygotes, but no homozygotes were found. Supplementary Fig. I shows the most relevant part of the pedigree. This sequence variation was not present in the remaining 94 probands with high HDL-C or in 200 normolipidemic unrelated control subjects.

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Molecular pathology of the CETP-IVS7+1 mutation

The CETP-IVS7+1 mutation disrupts the consensus splice donor site sequence of intron 7 (GT→TT). To delineate the molecular consequences of this mutation, we analyzed CETP mRNA isolated from peripheral white blood cells of an affected individual and a control subject. Using RT-PCR, control mRNA produced a single 212 bp fragment, indicating proper mRNA processing (Fig. 1A). However, an additional aberrant PCR product of ~150 bp was identified when using mRNA of the CETP-IVS7+1 carrier. DNA sequence analysis of this PCR product showed that the sequence of exon 6 was followed directly by exon 8, indicating that the mutation caused exon 7 to be skipped (61 bp) (Fig. 1B). Because of the resulting frameshift, translation of the aberrant mRNA is predicted to result in a truncated CETP protein of 196 amino acids (Fig. 1C). Using quantitative PCR, we assessed the concentration of the two CETP mRNA variants in adipose tissue of the affected individual. The estimated amount of mutant mRNA was 24.7 (arbitrary units), compared with a total of 93.3 (arbitrary units) of wild-type mRNA (both compared with GAPDH expression levels). These results indicate that 20% of the pool of CETP mRNAs consisted of the alternatively spliced mRNA product.

The ELISA used to determine plasma CETP concentration did not allow for the detection of the hypothesized truncated CETP protein, because it lacks the epitopes for the TP1 and TP2 antibodies that were used. To address whether this truncated product was synthesized and present in plasma of CETP-IVS7+1 carriers, another ELISA using monoclonal antibody ScFv 1CL8 directed against amino acids 68–87 and 110–129 of CETP was used (30). Data obtained with this antibody were not different.
from those obtained in the first assay, suggesting that there is no detectable truncated CETP protein present in fasted plasma of the carriers of this mutation. These data thus suggest that the CETP-IVS7+1 heterozygotes only have wild-type CETP protein in their circulation.

Heterozygosity for CETP-IVS7+1: effects on CETP and HDL-C

To carefully study the effects of the mutation in this family, the 25 heterozygotes for the CETP-IVS7+1 mutation were matched to 25 family controls of similar age, sex, and BMI (selected from 190 family members that were recruited). These 25 family controls presented with a lipid profile that was similar to that of a cohort of 2,381 healthy normolipidemic subjects from our database (for more information, see supplementary Table IV). This finding provided the basis for the use of the 25 family members as a control group in the analysis described below. The rationale for using these family controls is that lifestyle and genetic background affecting lipid parameters can be assumed to be similar in the family controls compared with those in family members carrying the CETP mutation. Three heterozygotes used statin therapy for proven cardiovascular disease; none of the control subjects received lipid-lowering medication. Illustrating the penetrance of this mutation, the heterozygotes presented with an average 40% decrease of plasma CETP concentration (0.94 ± 0.28 vs. 1.58 ± 0.31 μg/ml; P < 0.0001) (Table 3) and a concomitant mean 50% reduction in plasma CETP activity (40.1 ± 12.4 vs. 81.1 ± 20.4%; P < 0.0001) (Table 3) compared with family controls. Affected males (n = 13) and females (n = 12) showed nearly identical reductions of CETP activity and CETP concentration levels (40.6 ± 6.6 vs. 40.3 ± 18.9% and 0.96 ± 0.30 vs. 0.95 ± 0.26 μg/ml, respectively). Compared with family controls, HDL-C levels were strongly increased in the heterozygotes (2.06 ± 0.65 vs. 1.52 ± 0.39 mmol/l; P < 0.0001) (Table 3). Thus, in contrast with our finding in the entire HALP cohort that CETP was not associated with HDL-C levels, these data show that a profound loss of CETP activity as a result of partial CETP deficiency can underlie HALP.

Lipid metabolism in CETP-IVS7+1 heterozygotes, family controls, and 94 individuals with HALP

Next, we compared the 25 heterozygotes for CETP-IVS7+1 with individuals with HALP in which no CETP deficiency (n = 94) was identified. Although we sequenced the CETP gene only in individuals with low CETP concentrations, we here assumed the absence of CETP gene defects in the remaining subjects with CETP concentrations and CETP activity levels in the normal range. We used backward stepwise linear regression analysis, in which we adjusted for age, sex, BMI, smoking, and statin use when necessary. Exclusion of the individual receiving fibrate therapy did not affect the outcome. In the group with HALP (n = 94), HDL-C levels were significantly higher compared with the CETP-IVS7+1 heterozygotes (2.35 ± 0.43 vs. 2.06 ± 0.65 mmol/l; P = 0.04). Compared with controls, the increase of HDL-C levels in the HALP group was reflected by increases of both LpAI and LpAI:AII levels. However, the increase in LpAI was larger (+0.24 g/l) compared with that of LpAI:AII (+0.13 g/l). By contrast, heterozygosity for the CETP-IVS7+1 mutation had little and no significant effect on LpAI levels (0.57 ± 0.20 vs. 0.51 ± 0.13 g/l in controls; P = 0.23) but had a more profound and statistically significant effect on LpAI:AII levels (1.30 ± 0.25 vs. 1.11 ± 0.20 g/l in controls; P = 0.003). Although apoE concentration appeared higher in the HALP and CETP-IVS+1 groups, this did not reach statistical significance.

The HALP group further exhibited 40% lower triglyceride levels compared with the CETP-IVS7+1 heterozygotes (0.83 ± 0.39 vs. 1.37 ± 0.98 mmol/l; P < 0.0001) and higher LDL-C levels (3.58 ± 0.99 vs. 2.80 ± 1.02 mmol/l; P = 0.03). By contrast, apoB levels were not different be-

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Table 3. Demographic and clinical characteristics, plasma CETP concentration and activity, lipids, and apolipoproteins in unrelated subjects with HALP, heterozygotes for a novel CETP splice mutation (IVS7+1), and family controls

<table>
<thead>
<tr>
<th>Variable</th>
<th>Family Controls (n = 25)</th>
<th>CETP-IVS7+1 Heterozygotes (n = 25)</th>
<th>HALP (n = 94)</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (% male)</td>
<td>54.2</td>
<td>52</td>
<td>46.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>40 ± 22</td>
<td>39 ± 22</td>
<td>57.4 ± 13.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking (%)</td>
<td>19.0</td>
<td>20.0</td>
<td>19.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.3 ± 5.1</td>
<td>23.4 ± 4.9</td>
<td>24.0 ± 2.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CETP concentration (μg/ml)</td>
<td>1.58 ± 0.34</td>
<td>0.94 ± 0.28</td>
<td>1.90 ± 0.58</td>
<td>&lt;0.0001</td>
<td>0.01</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CETP activity (%)</td>
<td>81.1 ± 20.4</td>
<td>40.1 ± 12.4</td>
<td>99.9 ± 24.3</td>
<td>&lt;0.0001</td>
<td>0.005</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HDL-C (mmol/l)</td>
<td>1.52 ± 0.39</td>
<td>2.06 ± 0.65</td>
<td>2.35 ± 0.43</td>
<td>&lt;0.0001</td>
<td>0.001</td>
<td>0.04</td>
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<tr>
<td>ApoA1 (g/l)</td>
<td>1.63 ± 0.31</td>
<td>1.87 ± 0.42</td>
<td>1.98 ± 0.27</td>
<td>&lt;0.0001</td>
<td>0.11</td>
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<tr>
<td>LpAI (g/l)</td>
<td>0.51 ± 0.13</td>
<td>0.57 ± 0.20</td>
<td>0.75 ± 0.18</td>
<td>0.23</td>
<td>&lt;0.0001</td>
<td>0.004</td>
</tr>
<tr>
<td>LpAI:AII (g/l)</td>
<td>1.11 ± 0.20</td>
<td>1.30 ± 0.25</td>
<td>1.24 ± 0.18</td>
<td>0.03</td>
<td>0.02</td>
<td>0.18</td>
</tr>
<tr>
<td>ApoE (g/dl)</td>
<td>3.76 ± 0.97</td>
<td>4.12 ± 1.47</td>
<td>4.31 ± 1.88</td>
<td>0.36</td>
<td>0.16</td>
<td>0.67</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.23 ± 0.65</td>
<td>1.37 ± 0.98</td>
<td>0.83 ± 0.39</td>
<td>0.20</td>
<td>0.01</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LDL-C (mmol/l)</td>
<td>3.03 ± 1.07</td>
<td>2.80 ± 1.02</td>
<td>3.58 ± 0.99</td>
<td>0.27</td>
<td>0.13</td>
<td>0.05</td>
</tr>
<tr>
<td>ApoB (g/l)</td>
<td>1.16 ± 0.36</td>
<td>1.08 ± 0.40</td>
<td>1.12 ± 0.26</td>
<td>0.77</td>
<td>0.26</td>
<td>0.37</td>
</tr>
<tr>
<td>Total cholesterol (g/l)</td>
<td>5.07 ± 1.34</td>
<td>5.47 ± 1.18</td>
<td>6.31 ± 1.01</td>
<td>0.13</td>
<td>0.001</td>
<td>0.14</td>
</tr>
</tbody>
</table>

ApoA1, apolipoprotein A-I; LpAI, high density lipoprotein containing apolipoprotein A-I; LpAI:AII, high density lipoprotein containing apolipoprotein A-I and A-II. Values are given as means ± SD. P1, level of significance, CETP-IVS7+1 heterozygotes versus family controls; P2, level of significance, HALP versus family controls; P3, level of significance, HALP versus CETP-IVS7+1 heterozygotes. Correction was performed for differences in age, sex, smoking, BMI, and statin use when these parameters contributed significantly to the model. Exclusion of the individual receiving fibrate therapy did not affect the outcome.
between the groups, indicative of larger LDL particles in the HALP group. When comparing the CETP-IVS7+1 heterozygotes with controls, a slight reduction in LDL-C in the heterozygotes did not reach statistical significance, accompanied by the absence of differences in apoB concentration.

Thus, the CETP-IVS7+1 heterozygotes displayed an isolated high HDL-C phenotype that clearly differed from that of the HALP group, which exhibited a distinct high-HDL-C/low-triglyceride phenotype.

Other parameters
In the family with partial CETP deficiency, several additional analyses were carried out; these are summarized in Table 4. The CETP-IVS7+1 mutation did not affect plasma LCAT activity (88 ± 12 vs. 75 ± 20%; P = 0.15) and phospholipid transfer protein activity (113 ± 26 vs. 125 ± 26%; P = 0.21). Furthermore, NMR lipoprotein profiling in all 25 CETP-IVS7+1 heterozygotes and 20 family controls revealed increased HDL size in the heterozygotes (9.6 ± 0.6 vs. 9.2 ± 0.5 nm in controls; P = 0.008). The observed increase of HDL-C levels could be mainly accounted for by the accumulation of cholesterol in the large HDL particles. The apparent absence of an effect of CETP-IVS7+1 on plasma triglyceride levels in heterozygotes compared with controls (P = 0.20; Table 3) was reflected by similar triglyceride concentrations in large, intermediate, and small VLDLs (25.0 ± 27.6 vs. 35.0 ± 48.6 mg/dl (P = 0.20), 46.0 ± 31.7 vs. 47.8 ± 25.5 mg/dl (P = 0.81), and 8.5 ± 9.4 vs. 10.2 ± 6.8 mg/dl (P = 0.70), respectively). A trend toward an increased LDL size was observed in the CETP IVS7+1 heterozygotes compared with the controls (P = 0.09); levels of small as well as large LDL particles were not affected (P = 0.20 and 0.55, respectively).

DISCUSSION
In contrast to studies on HALP in Japan, this study shows that plasma CETP activity and CETP concentration are not related to HDL-C levels in 95 unrelated Caucasians with HALP. Screening for CETP mutations in this cohort led to the identification of a novel splice site defect (CETP-IVS7+1) in only one individual, indicating that CETP deficiency is rare among Caucasians of Dutch descent. Contrasting with the absence of a relation between CETP and HDL-C in the entire cohort, this CETP defect, causing a marked loss of CETP mass and activity, is associated with isolated high HDL-C in the family of this proband. This report describes for the first time in detail the effect of a CETP splice site mutation on lipid metabolism in Caucasians.

CETP and HALP
Our data clearly indicate that high HDL-C levels in Caucasian men and women with HALP are unlikely to result from differences in plasma CETP activity or CETP concentration. In fact, we noted a complete absence of correlations between HDL-C and CETP activity, CETP concentration, or CETP specific activities in this study. These findings agree with those from other studies in Caucasians without HALP (31, 32). Furthermore, population-based studies determining the contributions of common CETP gene variants, known to be associated with decreased plasma CETP concentrations, demonstrated that these polymorphisms explain only a small proportion of the observed variations of HDL-C in Caucasians (1, 32). However, these findings contrast with reports that CETP explains a large portion of HALP in Japan (17). Together, the current data lend support to the notion that CETP is not a major determinant of increased HDL-C levels in the general Caucasian population, not even in the setting of extremely high levels of HDL-C.

CETP deficiency in Caucasians
This study indicates that genetic CETP deficiency is rare among subjects with HALP in The Netherlands. Only 1 of 95 HALP families was identified with partial genetic CETP deficiency. This novel defect is shown to cause skipping of exon 7 and is predicted to result in a premature truncation of the CETP protein, a product that could not be detected in plasma of the carriers using a dedicated ELISA. The identification of this large family with partial CETP deficiency enabled us to analyze the effects of this disorder.

<table>
<thead>
<tr>
<th>Variable</th>
<th>CETP-IVS7+1 Heterozygotes (n = 25)</th>
<th>Family Controls (n = 25)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCAT activity (%)</td>
<td>88 ± 12</td>
<td>75 ± 20</td>
<td>0.15</td>
</tr>
<tr>
<td>Phospholipid transfer protein activity (%)</td>
<td>113 ± 26</td>
<td>125 ± 26</td>
<td>0.21</td>
</tr>
<tr>
<td>HDL size (nm)</td>
<td>9.6 ± 0.6</td>
<td>9.2 ± 0.5</td>
<td>0.008</td>
</tr>
<tr>
<td>Large HDL (μmol/l)</td>
<td>13.7 ± 6.3</td>
<td>9.0 ± 4.9</td>
<td>0.003</td>
</tr>
<tr>
<td>Small HDL (μmol/l)</td>
<td>20.6 ± 3.9</td>
<td>22.8 ± 2.8</td>
<td>0.04</td>
</tr>
<tr>
<td>VLDL triglyceride content</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large VLDL (mg/dl)</td>
<td>25.0 ± 27.6</td>
<td>35.0 ± 48.6</td>
<td>0.20</td>
</tr>
<tr>
<td>Intermediate VLDL (mg/dl)</td>
<td>46.0 ± 31.7</td>
<td>47.8 ± 25.5</td>
<td>0.81</td>
</tr>
<tr>
<td>Small VLDL (mg/dl)</td>
<td>8.5 ± 9.4</td>
<td>10.2 ± 6.8</td>
<td>0.70</td>
</tr>
<tr>
<td>LDL size (nm)</td>
<td>21.4 ± 0.9</td>
<td>21.0 ± 0.8</td>
<td>0.09</td>
</tr>
<tr>
<td>Large LDL (μmol/l)</td>
<td>472.2 ± 133.4</td>
<td>429.8 ± 228.7</td>
<td>0.55</td>
</tr>
<tr>
<td>Small LDL (μmol/l)</td>
<td>611.0 ± 445.7</td>
<td>810.6 ± 439.6</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Values are given as means ± SD; P values are for CETP-IVS7+1 heterozygotes versus family controls after correction for differences in age, sex, smoking, BMI, and statin use when these parameters contributed significantly to the model. None of the individuals received fibrate therapy.
on lipid metabolism in Caucasians and compare the ef-
fects with those in Japanese subjects with heterozygos-
ity for another CETP splice site defect, Int14+1 G→A (15).
Compared with their family controls, heterozygotes for
CETP-IVS7+1 or Int14+1 G→A present with 50% and
40% reductions of CETP activity, respectively, and conse-
sequential increases of HDL-C (35% and 75%, respectively)
(5). We have no explanation for the stronger effect on
HDL-C in Japanese subjects, but this might relate to dif-
fferences in lifestyle and genetic background. As seen in our
CETP-IVS7+1 heterozygotes, the Japanese counterparts
did not differ from their controls with respect to LDL-C,
apoB, and triglyceride levels, further emphasizing an iso-
lated high-HDL-C phenotype in partial CETP deficiency.
Interestingly, these findings differ from those identified
after inhibition of CETP activity using torcetrapib, a
pharmaceutical CETP inhibitor (12). In that study, a less
pronounced reduction of CETP activity (−38%) signifi-
cantly decreased baseline levels of LDL-C and triglycerides
by −17% and −18%, respectively, in individuals with low
HDL-C who had already received atorvastatin. In addition,
the effects of torcetrapib on lipoprotein sizes were stronger
compared with those observed in heterozygotes for CETP-
IVS7+1 with a 50% loss of CETP activity. Also, less pro-
ounced inhibition of CETP with JTT-705 in mildly
dyslipidemic individuals (up to −37%) showed significant
decrease of LDL-C levels (13). Apparently, the effects of life-
long endogenous loss of CETP as a result of CETP gene
mutations cannot be compared directly with the loss of
CETP activity induced by inhibitory compounds.

Partial CETP deficiency versus HALP without
CETP deficiency

Comparing the heterozygotes for CETP-IVS7+1 with the
remaining group of 94 individuals with HALP revealed
some interesting differences. In contrast to the CETP-
IVS7+1 heterozygotes, the HALP group exhibited a
marked 40% lower mean triglyceride level, for which we
have no direct explanation. Furthermore, HDL subfraction
analyses by means of rocket gel electrophoresis indicated
that the CETP-IVS7+1 mutation favors the occurrence of
LpAI:AII. There exists very little information in the current
literature on this topic. In one other study of partial CETP
deficiency, it was shown that LpAI and LpAI:AII levels were
not different between heterozygous CETP-deficient sub-
jects and controls (33), but only five affected individuals
were studied. Regarding LpAI and LpAI:AII fractions and
the risk of cardiovascular disease, data from epidemiolog-
ical studies are inconsistent, reporting reductions of LpAI
only (34, 35) as well as reductions of LpAI and LpAI:AII
(36–38) in coronary heart disease patients. Very recently,
Asztalos et al. (39) showed that neither LpAI nor LpAI:AII
was significantly associated with coronary heart disease
prevalence in The Framingham Offspring Study and the
placebo group of the Veterans Affairs HDL Intervention
Trial. Given the power of the latter studies (n = 1,019 and
741, respectively), it can be assumed that the different
concentrations in the respective HDL subfractions in our
study are unlikely to have an effect on atherosclerosis.

We also performed NMR lipoprotein subfraction analysis
in the family members with partial CETP deficiency.
Unfortunately, there exist no NMR data on HDL char-
acteristics in genetic CETP deficiency in the literature.
However, in agreement with studies using HPLC (18) or
gel electrophoresis (19) techniques in CETP-deficient
subjects, as well as observations from pharmacological
CETP inhibition (12), we observed increases of mean HDL
size and increased levels of large HDL particles. We also
identified a trend (P = 0.09) toward increased LDL size in
the CETP-IVS7+1 heterozygotes, as also observed in indi-
viduals treated with CETP inhibitors (12). Data on LDL
size derived from genetically CETP-deficient Japanese sub-
jects are limited to only a few studies with very small sample
sizes [n = 2 (20) and n = 5 (40)], again using techniques
other than NMR. Given this paucity of data, as well as the
observation of a trend toward statistical significance in our
study, the effects of genetic CETP deficiency on LDL-
related NMR parameters need further investigation.

Regarding the clinical consequences of these findings, it
is likely that the observed differences in lipid profiles of
the CETP-IVS7+1 heterozygotes may also translate into
differences in the risk of cardiovascular disease. In this
study, this is difficult to assess, given the small number of
heterozygotes and their low average age (39 ± 22 years).
Nevertheless, to investigate this, we measured carotid
intima media thickness in 67 of 94 HALP individuals, in 19
heterozygotes for the CETP-IVS7+1 mutation, and in 43
family controls (the latter group was expanded for this
imaging study). The data show no statistically significant
differences among the three groups (see supplementary
Fig. II), but the limited number of individuals does not
allow for firm conclusions.

Conclusions

This study shows that, overall, CETP does not play a major
role in defining HALP in Caucasians of Dutch descent.
However, heterozygosity for a novel CETP splice site mu-
tation, identified in one family, was associated with an iso-
lated high HDL-C level as the main lipid phenotype.

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REFERENCES

1. Boekholdt, S. M., O. W. Souverein, M. W. Tanck, G. K. Hovingh,
A. Kuivenhoven, R. I. Peters, H. Jansen, P. M. Schiffers, E. E. van
der Wall, P. A. Doevendans, et al. 2006. Common variants of mul-
tiple genes that control reverse cholesterol transport together ex-
plain only a minor part of the variation of HDL cholesterol levels.


