Improving cardiovascular disease prevention: from risk assessment to novel therapy
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Cholesteryl ester transfer protein and hyperalpha-lipoproteinemia in Caucasians

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ABSTRACT

It is unclear whether cholesteryl ester transfer protein (CETP) contributes to HDL cholesterol levels in hyperalphalipoproteinemia (HALP) in Caucasians. Moreover, even less is known about the effects of hereditary CETP deficiency in non-Japanese. We have studied 95 unrelated Caucasian individuals (46% male) with HDL cholesterol levels averaging 2.35 ± 0.42 mmol/L. Unlike findings in Japanese subjects with HALP, no correlations between CETP concentration or activity and HDL cholesterol were identified in our cohort. Screening for CETP gene defects in this cohort led to the identification of heterozygosity for a novel splice site mutation in one individual. CETP mRNA analysis of adipose tissue revealed that 20% of the CETP mRNA pool consisted of the anticipated alternatively spliced CETP mRNA. 25 heterozygotes for this mutation showed reductions of CETP concentration (-40%) and CETP activity (-50%), and a 35% increase of HDL cholesterol compared to family controls (matched for age, gender and body mass index). The heterozygotes presented with isolated high HDL cholesterol, while the remaining 94 HALP subjects exhibited a typical high HDL cholesterol and low triglyceride phenotype. The increase of HDL cholesterol in the CETP deficient heterozygotes was primarily due to elevated LpAI:AII levels, contrasting with a rise in both LpAI and LpAI:AII in the other group. This study suggests the absence of a relationship between CETP and HDL cholesterol levels in Caucasians with HALP. The data furthermore indicate that genetic CETP deficiency is rare amongst Caucasians and that this disorder presents itself with a phenotype that is different from subjects with HALP who have no mutation in the CETP gene.

Abbreviations – CETP, cholesteryl ester transfer protein; CETP-IVS7+1, G→T nucleotide substitution at nucleotide +1 of intron 7 of the CETP gene; HALP, hyperalphalipoproteinemia; HDL, high-density lipoprotein; LCAT, lecithin:cholesteryl acyltransferase; LDL, low-density lipoprotein; LpAI, HDL containing apolipoprotein AI only; LpAI:AII, HDL containing apolipoprotein AI and AII; PLTP, phospholipid transfer protein;
INTRODUCTION

In the general population, variations of HDL cholesterol levels are commonly ascribed to a combination of environmental and genetic factors (1). By contrast, extremely high levels of HDL cholesterol (hyperalphalipoproteinemia, HALP) are often the result of monogenetic disturbances. Among the genetic factors identified thus far (such as scavenger receptor B1 (2, 3) and apolipoprotein CIII 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 from HDL to apolipoprotein B containing lipoproteins (VLDL, IDL, LDL) (9, 10). Human CETP deficiency is accordingly characterized by cholesteryl ester enrichment of HDL which results in increased levels of HDL cholesterol. This observation has led to the development of small molecular inhibitors of CETP which have been shown efficacious to increase plasma levels of HDL cholesterol in humans (11-14).

The central role of CETP in human HDL metabolism only became evident after the identification of Japanese subjects suffering from genetic CETP deficiency. Homozygotes for loss of function mutations showed up to 4 times elevations of HDL cholesterol levels (15, 16). Since this discovery in 1989, CETP mutations have primarily been identified in Japan, and Maruyama et al. showed that genetic CETP deficiency is in fact a very frequent cause of HALP in this country (17). 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 HDL cholesterol levels compared to controls, respectively. Loss of CETP activity was furthermore characterized by increased HDL2 cholesterol concentration (18) and increased mean HDL particle size (19). Taken the central role of CETP in exchanging neutral lipids between HDL and apolipoprotein B 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 suffer from changes in LDL cholesterol, apolipoprotein B or triglyceride levels (5), despite an approximate 50% reduction of CETP activity levels. Regarding LDL particle size, it has been shown that the dimensions of these particles are distributed over an atypical wide range in subjects suffering from homozygous CETP deficiency (20), but data covering this specific issue are scarce.

Taken together, genetic CETP deficiency and the use of CETP inhibitors have illustrated 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 mainly derived from subjects living in Japan. In Caucasians, the literature on CETP and HALP is scarce. In fact, only a few Caucasian individuals suffering from CETP deficiency have been presented in case reports (21-24). Consequently, it is not known whether CETP plays a major role in determining HDL cholesterol levels in non-Japanese subjects. The first objective of this study was therefore to investigate the relationship between CETP and HDL cholesterol levels in Caucasian subjects with
HALP. The second objective was to screen for genetic CETP deficiency in this study 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 study group*

Over the past decade, we have used our lipid clinic network and contacts with general practitioners in the Netherlands to collect plasma and DNA of individuals with HALP, with the intent to identify novel genes that control HDL cholesterol levels. The vast majority of these individuals came to the attention of their physicians through general lipid tests which revealed high HDL cholesterol levels. For the present study, we studied 95 unrelated Caucasian index subjects (44 males, 51 females) from families in which HALP was established in at least 3 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 cholesterol levels above the 90th percentile for age and gender at 2 visits, without the presence of secondary causes that could lead to a HALP phenotype (extensive regular aerobic exercise, regular substantial alcohol intake, estrogen replacement therapy, drugs (fibrates, nicotinic acid, phenytoin)). Nine out of the 95 subjects received statin therapy for suspected (n=1) or proven (n=4) cardiovascular disease or for unknown reason (n=4). One of these subjects was additionally treated with a fibrate (Gemfibrozil), but the presence of a HALP phenotype before start of this therapy as well as the presence of this phenotype in 3 first degree relatives were reason for inclusion of this individual.

Informed consent was obtained for blood sampling and genetic analyses, and the study was approved by the Medical 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 concentration <1.4 μg/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 concentration in these subjects co-segregated 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 base pairs 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-IVS7+1 mutation was performed by restriction fragment length polymorphism analysis using the forward 5'-CGGTGCCTGGTACACACTAG-3' and reverse 5'-CATAGTGCATCAGGTGGCTT-3' primers for PCR and digestion with XcmI (New England Biolabs, Beverly, MA, USA), which digests wild-type (wt) DNA, but not the mutant sequence.

**RNA isolation and reverse transcription PCR**

Total RNA was isolated from peripheral leukocytes. One volume of whole blood was mixed with 9 volumes lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA) and kept on ice for 15 minutes. Cells were collected by centrifugation at 3000g, washed twice by resuspension in lysis buffer and repeated centrifugation. The pellet was resuspended in Tripture and RNA was isolated according to the manufacturer’s instructions (Roche Diagnostics Nederland BV, Almere, The Netherlands). First strand cDNA was generated from 1μg total RNA with Superscript II Rnase H⁻ (Invitrogen, Breda, The Netherlands) with an oligo dT14VN 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'-TGATGGGACTCCAGGTAGGA-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, Almere, The Netherlands) from abdominal adipose tissue obtained by needle aspiration from a heterozygote for CETP-IVS7+1 and 2 control subjects. The amount of total CETP mRNA (wild type & mutant) and wild type CETP mRNA was determined with LightCycler SybrGreen quantification using primer sets in exons 2, 3 and exons 6, 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 apolipoproteins A-I, A-II, B and E were measured by nephelometry (Dade Behring, Marburg, Germany). LpAI and LpAI:All concentrations were determined by rocket gel electrophoresis (Sebia, France). Plasma CETP, LCAT and phospholipid transfer protein (PLTP) 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, USA). A P-value <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 cholesterol. Linear regression analysis was performed to examine these relationships adjusted for age, sex, body mass index, smoking and statin/fibrate use when these factors significantly contributed to the regression 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, body mass index, smoking and statin use when these parameters significantly contributed to the model. To investigate the effect of fibrate therapy, analyses were also performed following exclusion of the individual receiving Gemfibrozil.

**RESULTS**

**CETP and hyperalphalipoproteinemia**

The first objective of this study was to investigate the relationship between CETP and HDL cholesterol levels in Caucasian subjects with HALP. To this purpose, we studied 95 unrelated probands from families in which HALP was established in at least 3 first degree relatives. The average HDL cholesterol level in this group was 2.35 ± 0.42 mmol/L (table 1), with higher HDL cholesterol levels in women compared to 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 non-significant lower CETP concentration compared to the 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%).

**TABLE 1. Demographic and clinical characteristics, lipids, CETP concentration, CETP activity and specific CETP activity in 95 individuals with hyperalphalipoproteinemia**

<table>
<thead>
<tr>
<th></th>
<th>All  n=95</th>
<th>Men n=44</th>
<th>Women n=51</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>57 ± 14</td>
<td>57 ± 14</td>
<td>58 ± 14</td>
<td>-</td>
</tr>
<tr>
<td>Smoking, %</td>
<td>20.0</td>
<td>20.5</td>
<td>19.6</td>
<td>-</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>24.0 ± 2.7</td>
<td>24.2 ± 2.6</td>
<td>23.8 ± 2.8</td>
<td>-</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>6.31 ± 1.01</td>
<td>6.25 ± 1.05</td>
<td>6.37 ± 0.98</td>
<td>-</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>3.58 ± 0.98</td>
<td>3.80 ± 0.97</td>
<td>3.39 ± 0.97</td>
<td>-</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>2.35 ± 0.42</td>
<td>2.06 ± 0.33</td>
<td>2.61 ± 0.32</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>0.83 ± 0.39</td>
<td>0.85 ± 0.49</td>
<td>0.81 ± 0.29</td>
<td>-</td>
</tr>
<tr>
<td>CETP concentration, μg/mL</td>
<td>1.89 ± 0.60</td>
<td>1.79 ± 0.54</td>
<td>1.98 ± 0.60</td>
<td>0.21</td>
</tr>
<tr>
<td>CETP activity, %*</td>
<td>99.0 ± 25.4</td>
<td>96.8 ± 19.4</td>
<td>100.9 ± 29.7</td>
<td>0.42</td>
</tr>
<tr>
<td>Specific CETP activity, %/μg</td>
<td>54.3 ± 12.8</td>
<td>56.9 ± 13.2</td>
<td>52.0 ± 12.2</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Values are given as means ± standard deviation; P: men vs. women, following correction for differences in age, sex, smoking, body mass index and statin use when these parameters significantly contributed to the model. Exclusion of the individual receiving fibrate therapy did not affect outcome.

*Assessed in 71 of 95 subjects (males: 33/44; females: 38/51) without selection bias.
Mean CETP activity was slightly lower in men (96.8 ± 19.4%) compared to women (100.9 ± 29.7%), but this difference was not statistically significant (P=0.42). Thus, compared to men, the women presented with higher HDL cholesterol levels accompanied by non-significant elevations in CETP concentration and activity (exclusion of the individual receiving fibrate therapy generated similar results).

Using the data of 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 cholesterol levels were not correlated with either 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, body mass index, smoking and statin/fibrate use when necessary, generated similar results. CETP activity was assessed in 71 out of 95 subjects due to a lack of sufficient plasma.

### Table 2. Relationships between CETP activity, CETP concentration, specific CETP activity and HDL cholesterol in 95 subjects with HDL cholesterol levels above the 90th percentile for age and sex

<table>
<thead>
<tr>
<th></th>
<th>r</th>
<th>P1</th>
<th>β</th>
<th>P2</th>
</tr>
</thead>
<tbody>
<tr>
<td>CETP activity* – 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* – HDL cholesterol</td>
<td>0.17</td>
<td>0.16</td>
<td>0.05</td>
<td>0.66</td>
</tr>
<tr>
<td>CETP concentration – HDL cholesterol</td>
<td>0.14</td>
<td>0.17</td>
<td>0.11</td>
<td>0.30</td>
</tr>
<tr>
<td>Specific CETP activity* – HDL cholesterol</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; β: standardized coefficient derived from linear regression analysis adjusting for age, sex, smoking, body mass index and statin/fibrate use when these parameters significantly contributed to the model, P2: level of significance.

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

### 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 concentration <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 concentration in these subjects co-segregated 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 concentration). This analysis revealed a number of CETP gene variations that were either previously described polymorphisms (28, 29), or were not expected to affect CETP gene transcription, based on their positions in non-regulatory promoter or intronic sequences. We did not further analyze these variations, 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 cholesterol 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 materials and methods section for details). This led to the identification of 24 additional heterozygotes, but no homozygotes were found. The penetrance of the CETP-IVS7+1
mutation with respect to CETP concentration and HDL cholesterol levels was very high: 23 out of 25 carriers (92%) had CETP concentration below 1.4 μg/mL (range 0.58-1.18 μg/mL) and 16 out of 25 subjects (68%) had HDL cholesterol above the 90th percentile adjusted for age and sex.

**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 base pairs fragment, indicating proper mRNA processing (see figure 1A). However, an additional aberrant PCR product of approximately 150 base pairs 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 directly followed by exon 8, indicating that the mutation caused skipping of exon 7 (61 base pairs) (figure 1B). Due to the resulting frameshift, translation of the aberrant mRNA is predicted to result in a truncated CETP protein of 196 amino acids (figure 1C).

Using quantitative PCR, we assessed the concentration of the 2 CETP mRNA variants in adipose tissue of the affected individual. The estimated amount of mutant mRNA was 24.7 (arbitrary units), compared to a total of 93.3 (arbitrary units) wild type mRNA (both compared to GAPDH expression levels). These results indicate that 20% of the pool of CETP mRNAs consisted of the alternatively spliced mRNA product.

**FIGURE 1.** Molecular characterization of the CETP-IVS7+1 defect. (A) RT-PCR on RNA of a CETP-IVS7+1 carrier and control subject. Plasmid CETP cDNA was used as control. Primers were located in exon 6 and exon 8. Control mRNA produces a 212 base pairs fragment indicating proper processing; an additional fragment is present in mRNA of the IVS7+1 carrier. (B) Partial DNA sequence of the mutant RT-PCR product (151 base pairs) of the IVS7+1 carrier showing skipping of exon 7. (C) Hypothetical effects of the frameshift caused by exon skipping on translation of the mutant CETP mRNA. In brackets total length of wild type and mutant CETP protein in amino acids (wt= wild type; mut= mutant).
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 employed. To address whether this truncated product was synthesized and present in plasma of CETP-IVS7+1 carriers, another ELISA using a 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 cholesterol
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 body mass index (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 (data not shown). This finding provided the basis for use of the 25 family members as a control group in this analysis described below. The rationale for using these family controls is that lifestyle and genetic background affecting lipid parameters can be assumed similar in the family controls compared to 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 on average 40% decrease of plasma CETP concentration (0.94 ± 0.28 μg/mL 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 to family controls. Affected males (n=13) and females (n=12) showed nearly identical reductions of CETP activity and CETP concentration levels (40.0% ± 6.6 vs. 40.3% ± 18.9 and 0.96 ± 0.30 μg/mL vs. 0.93 ± 0.26 μg/mL, respectively). Compared to family controls, HDL cholesterol levels were strongly increased in the heterozygotes (2.06 ± 0.65 mmol/L vs. 1.52 ± 0.39 mmol/L; P<0.0001; table 3). Thus, contrasting with our finding in the entire HALP cohort that CETP was not associated with HDL cholesterol 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
This section compares 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 concentration, we here assumed the absence of CETP gene defects in the remaining subjects with CETP concentration and CETP activity levels in the normal range. We used backward stepwise linear regression analysis, in which we adjusted for age, sex, body mass index, smoking and statin use when necessary. Exclusion of the individual receiving fibrate therapy did not affect outcome. In the group with HALP (n=94), HDL cholesterol levels were
### 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 site mutation (IVS7+1) and family controls.

<table>
<thead>
<tr>
<th></th>
<th>Family controls</th>
<th>CETP-IVS7+1 HE</th>
<th>HALP</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=25</td>
<td>n=25</td>
<td>n=94</td>
<td></td>
<td></td>
<td></td>
</tr>
<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>Body mass index, 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 ± 0.31</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 cholesterol, 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>&lt;0.0001</td>
<td>0.04</td>
</tr>
<tr>
<td>Apolipoprotein A-I, g/L</td>
<td>1.63 ± 0.31</td>
<td>1.87 ± 0.42</td>
<td>1.98 ± 0.27</td>
<td>0.007</td>
<td>&lt;0.0001</td>
<td>0.11</td>
</tr>
<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>0.001</td>
</tr>
<tr>
<td>LpAI:AlII, g/L</td>
<td>1.11 ± 0.20</td>
<td>1.30 ± 0.25</td>
<td>1.24 ± 0.18</td>
<td>0.003</td>
<td>0.02</td>
<td>0.18</td>
</tr>
<tr>
<td>Apolipoprotein E, 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 cholesterol, 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>0.03</td>
</tr>
<tr>
<td>Apolipoprotein B, 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, mmol/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>

Values are given as means ± standard deviation; P1: CETP-IVS7+1 HE vs. family controls; P2: HALP vs. family controls; P3: HALP vs. CETP-IVS7+1 HE. Correction was performed for differences in age, sex, smoking, body mass index and statin use when these parameters significantly contributed to the model. Exclusion of the individual receiving fibrate therapy did not affect outcome. Abbreviations: HALP, hyperalphalipoproteinemia; HE, heterozygotes.

significantly higher compared to the CETP-IVS7+1 heterozygotes (2.35 ± 0.43 mmol/L vs. 2.06 ± 0.65 mmol/L; P=0.04). Compared to controls, the increase of HDL cholesterol levels in the n=94 group was reflected by increases of both LpAI and LpAI:AlII levels. However, the increase in LpAI was larger (+0.24 g/L) compared to LpAI:AlII (+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 g/L vs. 0.51 ± 0.13 g/L in controls; P=0.23) but had a more profound and statistically significant effect on LpAI:AlII levels (1.30 ± 0.25 g/L vs. 1.11 ± 0.20 g/L in controls; P=0.003). Although apolipoprotein E concentration appeared higher in the n=94 and the CETP-IVS+1 groups, this did not reach statistical significance.

The n=94 group further exhibited 40% lower triglyceride levels compared to the CETP-IVS7+1 heterozygotes (0.83 ± 0.39 mmol/L vs. 1.37 ± 0.98 mmol/L; P<0.0001) and higher LDL cholesterol levels (3.58 ± 0.99 mmol/L vs. 2.80 ± 1.02 mmol/L; P=0.03). By contrast, apolipoprotein B levels were not different between the groups, indicative of larger LDL particles in the n=94 group. When comparing the CETP-IVS7+1 heterozygotes with controls, a slight reduction in LDL cholesterol in the heterozygotes did not reach statistical significance, accompanied by the absence of differences in apolipoprotein B concentration.

Taken together, the CETP-IVS7+1 heterozygotes displayed an isolated high HDL cholesterol phenotype which clearly differed from the n=94 group that exhibited a distinct high HDL cholesterol and low triglyceride phenotype.
Other parameters

In the family with partial CETP deficiency, several additional analyses have been carried out which 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 PLTP 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 nm vs. 9.2 ± 0.5 nm in controls; P=0.008). The observed increase of HDL cholesterol 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 as compared to controls (P=0.20; table 3) was reflected by similar triglyceride concentrations in large, intermediate and small sized VLDL (25.0 ± 27.6 mg/dL vs. 35.0 ± 48.6 mg/dL, P=0.20; 46.0 ± 31.7 mg/dL vs. 47.8 ± 25.5 mg/dL, P=0.81 and 8.5 ± 9.4 mg/dL vs. 10.2 ± 6.8 mg/dL, P=0.70, respectively). A trend towards increased LDL size was observed in the CETP-IVS7+1 heterozygotes as 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).

**TABLE 4. Lipoprotein modifying enzymes and NMR lipoprotein profiling in heterozygotes for a novel CETP splice site mutation (IVS7+1) and family controls**

<table>
<thead>
<tr>
<th></th>
<th>CETP-IVS7+1 HE 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>PLTP 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 ± 443.7</td>
<td>810.6 ± 439.6</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Values are given as means ± standard deviation; P: CETP-IVS7+1 HE vs. family controls, following correction for differences in age, sex, smoking, body mass index and statin use when these parameters significantly contributed to the model. None of the individuals received fibrate therapy. Abbreviations: HE, heterozygotes; LCAT, lecithin:cholesteryl acyltransferase; PLTP, phospholipid transfer protein

**DISCUSSION**

In contrast to studies on HALP in Japan, this study shows that plasma CETP activity and CETP concentration are not related to HDL cholesterol 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 cholesterol in
the entire cohort, this CETP defect – causing a marked loss of CETP mass and activity – is associated with isolated high HDL cholesterol 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 hyperalphalipoproteinemia**

Our data clearly indicate that high HDL cholesterol levels in Caucasian men and women with HALP are unlikely to result from differences in plasma CETP activity or CETP concentration. We in fact noted a complete absence of correlations between HDL cholesterol 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 contribution of common CETP gene variants, known to be associated with decreased plasma CETP concentration, demonstrated that these polymorphisms explain only a small proportion of the observed variations of HDL cholesterol in Caucasians (1, 32). However, these findings contrast with reports describing that CETP explains a large portion of HALP in Japan (17). Taken together, the current data lend support to the notion that CETP is not a major determinant of elevated HDL cholesterol levels in the general Caucasian population, not even in a setting of extremely high levels of HDL cholesterol.

**CETP deficiency in Caucasians**

This study indicates that genetic CETP deficiency is rare amongst subjects with HALP in The Netherlands. Only one out of 95 HALP families was identified with partial genetic CETP deficiency. The respective novel defect is shown to cause skipping of exon 7 and 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 to analyze the effects of this disorder on lipid metabolism in Caucasians and compare the effects to Japanese subjects suffering from heterozygosity for another CETP splice site defect (Int14+1 G→A (15)). Compared to their family controls, heterozygotes for CETP-IVS7+1 or Int14+1 G→A present with a 50% and 40% reduction of CETP activity, respectively, and a consequential increase of HDL cholesterol (35% and 75%, respectively) (5). We have no explanation for a stronger effect on HDL cholesterol in the Japanese subjects, but this might relate to differences 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 cholesterol, apolipoprotein B and triglyceride levels, further underlining an isolated high HDL cholesterol phenotype in partial CETP deficiency. Interestingly, these findings differ from those identified after inhibition of CETP activity when using torcetrapib, a pharmaceutical CETP inhibitor. Here, a less pronounced reduction of CETP activity (-38%) significantly dropped baseline levels of LDL cholesterol and triglycerides by -17 and -18%, respectively in individuals with low HDL cholesterol who already received atorvastatin (12).
addition, the effects of torcetrapib on lipoprotein sizes were stronger as compared to those observed in heterozygotes for CETP-IVS7+1 with a 50% loss of CETP activity. Also, less pronounced inhibition of CETP with JTT-705 in mildly dyslipidemic individuals (up to -37%) showed significant decrease of LDL cholesterol levels (13). Apparently, the effects of life-long endogenous loss of CETP due to CETP gene mutations cannot be directly compared to 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 former group exhibited a marked 40% lower mean triglyceride level, for which we have no direct explanation. HDL subfraction analysis by means of rocket gel electrophoresis furthermore indicated that the CETP-IVS7+1 mutation favors the occurrence of HDL particles that contain both apolipoprotein A-I and A-II (LpAI:AII). There exists very little information in the current literature on this topic. In one other study on partial CETP deficiency it was shown that LpAI and LpAI:AII levels were not different between heterozygous CETP deficient subjects and controls (33), but here only 5 affected individuals were studied. Regarding LpAI and LpAI:AII fractions and risk of cardiovascular disease, data from epidemiological 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. Asztalos showed that neither LpAI nor LpAI:AII were significantly associated with coronary heart disease prevalence in The Framingham Offspring Study and the placebo group of the VA-HIT trial (39). Taken the power of the latter studies (n=1,019 and 741, respectively), it can thus be assumed that the different concentrations in the respective HDL subfractions in the present 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 characteristics in genetic CETP deficiency in the literature. However, in line with studies using HPLC (18) or gel electrophoresis (19) techniques in CETP deficient subjects, as well as observations following pharmacological CETP inhibition (12), we observed increases of mean HDL size and increased levels of large HDL particles. Besides, we also identified a trend (P=0.09) towards increased LDL size in the CETP-IVS7+1 heterozygotes as also observed in individuals treated with CETP inhibitors (12). Data on LDL size derived from genetically CETP deficient Japanese are limited to only a few studies with very small sample size (n=2 (20, 40)), again using other techniques than NMR. Given this paucity of data, as well as the observation of a trend towards statistical significance in our study, the effects of genetic CETP deficiency on LDL related NMR parameters needs further investigation.

Regarding the clinical consequences of all these findings, it is likely that the observed differences in lipid profiles of the CETP-IVS7+1 heterozygotes may also translate into differences in risk of cardiovascular disease. In the present study, this is hard to assess, given the small number of
heterozygotes as well as their low average age (39 ± 22 years). To nevertheless investigate this, we measured carotid intima media thickness (IMT) 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 amongst the 3 groups, 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 hyperalphalipoproteinemia in Caucasians of Dutch descent. However, heterozygosity for a novel CETP splice site mutation, identified in one family, was associated with isolated high HDL cholesterol as the main lipid phenotype.

Acknowledgements
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References
CETP and hyperalphalipoproteinemia