The Role of the ABCA1 Transporter and Cholesterol Efflux in Familial Hypoalphalipoproteinemia

Short title: ABCA1 and Familial Hypoalphalipoproteinemia

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ABSTRACT

Objective. Defects in the gene encoding for the ATP Binding Cassette A1 (ABCA1) transporter were shown to be one of the genetic causes for familial hypoalphalipoproteinemia (FHA). We investigated the role of ABCA1 mediated cholesterol efflux in Dutch subjects suffering from FHA. Methods and Results: Eighty-eight subjects (mean HDL-c levels 0.63 ± 0.21 mmol/L) were enrolled. Fibroblasts were cultured and loaded with 3H-cholesterol. ABCA1 and non-ABCA1 mediated efflux was studied by using apoA-I, HDL and methyl-β-cyclodextrin as acceptors. Efflux to apoA-I was decreased in four patients (4/88, 4.5 %) and in all cases a mutation in the ABCA1 gene was found. In the remaining 84 subjects no correlation between efflux to apoA-I and HDL-c was found. Efflux to both HDL and cyclodextrin, in contrast, did correlate with HDL-c plasma levels (r=0.34; p=0.01 and r=0.27; p=0.008 respectively). Conclusions: The prevalence of defects in ABCA1-dependent cholesterol efflux in Dutch FHA patients is low. The significant correlation between plasma HDL-c levels and methyl-β-cyclodextrin mediated efflux in the FHA patients with normal ABCA1 function suggests that non-ABCA1 mediated efflux might also be important for plasma HDL-c levels in these individuals.

KEY WORDS:
HDL, ABCA1, efflux, cholesterol, Familial hypoalphalipoproteinemia
INTRODUCTION

Decreased plasma levels of high-density lipoprotein cholesterol (HDL-c) have consistently been shown to be associated with an increased risk for coronary artery disease (CAD).

The atheroprotective role of HDL-c is in part ascribed to its capacity to transport cholesterol from peripheral cells (including vascular macrophages) to the liver, a mechanism known as Reverse Cholesterol Transport (RCT) (1).

A considerable number of proteins, enzymes and receptors are involved in RCT and in principle all may play a role in the pathogenesis of atherosclerosis. Identification of novel genes and proteins in the pathway may be accomplished by family studies and genetic analysis. By means of this strategy, a crucial rate-controlling step in RCT has been identified by performing linkage analysis and mutation detection in families with FHA, not caused by LCAT or apoA-I mutations (2-4). The culprit gene was shown to encode a 250kD transmembrane protein called ABCA1. ABCA1 belongs to the family of ATP Binding Cassette (ABC) proteins and is expressed ubiquitously in human tissues (5). In peripheral cells, including vascular macrophages, ABCA1 regulates energy dependent transport of cholesterol and phospholipids to apoA-I, the major protein in HDL. Mutations in the gene encoding ABCA1 give rise to decreased cholesterol and phospholipid efflux to apoA-I (6-8). Homozygosity for mutations in the ABCA1 gene causes Tangier Disease (TD) and heterozygosity is one of the causes for Familial Hypoalphalipoproteinemia (FHA). TD is characterized by near absence of plasma HDL-c, whereas in ABCA1 heterozygotes, HDL-c levels are approximately half of the normal levels for sex and age. A strong correlation exists between cholesterol efflux capacity from fibroblasts and plasma HDL-c levels in ABCA1 homo- and heterozygous patients (9).

The role of ABCA1 dysfunction in CAD risk has been the subject of debate. Before the molecular defect underlying TD was identified and the disease was diagnosed by clinical
assessment, CAD was found to occur in less than half of cases over 35 years of age (10), which is a remarkably low incidence given the severity of HDL deficiency. It has been suggested that this relative protection from atherosclerosis in TD patients is caused by the low levels of atherogenic low-density lipoprotein cholesterol (LDL-c) (11). Due to the small numbers of TD patients, the strong referral bias and the prior inability to diagnose this disorder by genetic screening, however, it has been so far impossible to draw firm conclusions regarding the risk for CAD in TD patients. More recently, a threefold higher prevalence of symptomatic vascular disease was found in heterozygotes for ABCA1 (12). We have also described a strong correlation between cholesterol efflux and CAD risk, as measured by means of the surrogate marker, Intima Media Thickness (IMT), in ABCA1 heterozygotes (9). In line with these findings, ABCA1 knockout mice were shown to suffer from enhanced atherosclerosis and in contrast overexpression of ABCA1 protected against diet-induced atherosclerosis. (13;14) These findings further confirmed our observations in humans that ABCA1 dysfunction does indeed promote atherogenesis.

An important unanswered question is how often dysfunctional ABCA1 mediated efflux underlie FHA.

Mott and colleagues reported that ABCA1-mediated cholesterol efflux was reduced in 50 percent of the tested 14 probands. An ABCA1 mutation was found in 4 of those cases, suggesting that 29% (4/14) of these Canadian patients suffered from FHA due to ABCA1 mutations (15).

The aim of the present study was to establish the prevalence of ABCA1 mediated cholesterol efflux defects in a cohort of Dutch patients with familial low HDL. We therefore measured in the fibroblasts of our large FHA cohort, the cholesterol efflux to apoA-I, reflecting ABCA1 mediated efflux and to cyclodextrin and HDL, reflecting non-ABCA1 mediated efflux. DNA
from all patients with defects in ABCA1 mediated efflux was subjected to sequencing of the ABCA1 gene.

**METHODS**

**Patients**

Patients were recruited from the Vascular Research Network in the Netherlands and referred to the Academic Medical Center, Amsterdam. The main inclusion criterion was that the patient should have an isolated low HDL-c level (below the fifth percentile corrected for age and sex). FHA was defined as the presence of a similar lipid abnormality in at least one first-degree family member. Informed consent was obtained from all subjects for plasma sampling, storage and genetic analysis, under a protocol approved by the Ethics Committee of the Academic Medical Center in Amsterdam.

**Lipids and Lipoproteins**

Blood samples were collected in EDTA-containing tubes from all participants following an overnight fast. Plasma total cholesterol was measured with an enzymatic colorimetric procedure (CHOD-PAP, Boehringer Mannheim, Mannheim Germany) as previously described (16). HDL-cholesterol was determined after precipitation of apoB containing lipoproteins with phosphotungstic acid and magnesium. LDL-cholesterol was calculated using the Friedewald formula (17). Triglycerides were measured using an enzymatic colorimetric method using lipase, glycerol kinase and glycerol-3-phosphate 3 oxidase.

**Efflux studies**

Fibroblasts were obtained from participants by explant culture from a 3mm punch biopsy at a 1mm skin thickness and were cultured (until passage 5-15) in 24 wells culture plates full
confluency, essentially as described earlier (9). The culture medium was DMEM supplemented with 10% v/v FCS. After washing the cells with DMEM, they were loaded with 30 microg/ml $^3$H-cholesterol (38 Ci/mm mol) in DMEM during 24 hours. After washing the cells 5 times with PBS /BSA 0.2% (w/v), the efflux assay was started by adding 5 µg/ml ApoA-I, 50 µg/ml HDL3 or 1mM methyl-ß-cyclodextrin to the wells. After 20 hours incubation at 37°C the medium was collected and centrifuged. Subsequently, $^3$H-cholesterol was quantified by liquid scintillation counting. Total cellular $^3$H-cholesterol was determined after extraction of the cells with 2-propanol. The percentage efflux was calculated by dividing the radioactive counts in the efflux medium by the sum of the counts in the medium and the cell extract. Efflux to apoA-I and HDL was linear over the time span of the experiment (data not shown).

Normal values for efflux to the different acceptors were determined in fibroblasts derived from a group of 11 healthy normolipidemic volunteers. Plasma HDL-c levels in those subjects were shown to be above the 15th percentile corrected for age and sex.

To test the effect of the passage number on cholesterol efflux, fibroblasts of five controls were cultured to passage 9 to 20 and cholesterol efflux was measured. No significant effect of passage number on efflux was discerned.

Mean efflux to apoA-I in this control group was 9.86 % of total cholesterol in 20 hours. (SD 2.71% (95%CI), range; 6.49-14.31%). Efflux to apoA-I was considered to be abnormal at a value below 4.45% (= mean efflux in controls minus 2SD).

Mutation detection and Sequence Analysis

Leucocytes were isolated from buffy coat for DNA extraction. For mutation detection, all forward and reverse PCR primers, flanking each exon, were designed with Repeat Master (http://ftp.genome.washington.edu/cgi-bin/Repeat Master) and Primer3 (http://www-
genome.wi-mit.edu/genome_software/other/primer3.html). The PCR protocol has been previously described (16).

**Statistical analysis**

Lipid values are displayed as mean ± SD. Student’s t test for unpaired data was used to compare individuals with ABCA1 defects with those not having defective apoA-I mediated efflux. A linear regression model was used for the correlation analysis between plasma HDL-c level and cholesterol efflux to the different acceptors. The Hotelling t-test was used to test the difference in correlations between HDL-c plasma level and HDL-apoA-I efflux and cyclodextrin efflux. All data was statistically analyzed using SPSS software.

**RESULTS**

Over the course of three years, one hundred and six (106) patients with HDL-c levels below the 5th percentile for sex and age were identified. Eighteen (18) patients were excluded from further study based on the absence of low HDL in a first-degree family member. Characteristics of the remaining eighty-eight (88) patients enrolled in this study are summarized in table 1. A skin biopsy was performed in all participants and fibroblasts were cultured. After growth to confluency, the cholesterol efflux to apoA-I, rHDL or the aspecific acceptor methyl-β-cyclodextrin was measured. Efflux to apoA-I was found to be significantly decreased in only 4 patients (4.5%). Genetic analysis revealed these patients were either compound heterozygotes or heterozygotes for mutations in the gene encoding for ABCA1. The two compound heterozygous patients have been described previously (one of the compound heterozygous carriers suffered from a missense mutation (T to C at position 4369) resulting in a C1477R, and a defect (IVS24 + 1G to C) that caused differential splicing, whereas the other was shown to carry a missense mutation (C to T at position 3181 resulting
in T929I) and a de novo non-sense mutation (16). A missense mutation (T3212C) resulting in M1091T, and a C to T nucleotide substitution at position 6844 resulting in P2150L were the defects in the two heterozygous carriers. Thirteen patients had very low levels of plasma HDL-c levels (<0.5 mmol/L) and normal cholesterol efflux to apoA-I, indicating that other factors, besides ABCA1, must be responsible for such isolated low HDL in these subjects. In six of these 13 patients we discovered a novel mutation in the gene encoding apoA-I (mutation L178P; manuscript in preparation). In addition, another one of these 13 patients had two defects in the LCAT gene (unpublished data).

Efflux to apoA-I of all remaining 84 patients was in the normal range (4.45-14.3%).

To assess the relationship between efflux to other cholesterol acceptors and plasma HDL-c levels, efflux measurements to various acceptors were plotted against HDL-c levels.

Figure 1 shows the correlation between apoA-I efflux and plasma HDL-c levels in the entire study population. Interestingly, no correlation between the two parameters is evident. However, efflux to HDL was significantly (r=0.34; p=0.001) correlated with serum HDL-c levels (Fig. 2). This correlation may be partially due to the presence of patients with ABCA1 mutations. Nevertheless, when this analysis was repeated excluding these four patients, the correlation between HDL-c and efflux to HDL remained significant (r=0.26, p=0.05).

By subtracting apoA-I-mediated efflux from HDL-mediated efflux, the influence of ABCA1 independent processes may be evaluated. As depicted in Fig. 3 this residual efflux also correlated significantly with plasma HDL-C in our patients (r=0.32 p=0.008). In contrast, there was no correlation between efflux to HDL and total cholesterol or triglycerides. The relation between efflux to methyl-β-cyclodextrin, an independent method to monitor non-specific cholesterol efflux, and plasma levels of HDL-c were significantly correlated (r=0.27, p=0.008). The correlation values for HDL minus apoA-I efflux and cyclodextrin efflux did not differ significantly (Hotelling t-test).
DISCUSSION

In our study we determined the prevalence of defects in ABCA1 mediated cellular cholesterol efflux in a large cohort of Dutch patients with isolated low HDL-c. We show that such defects account for a small percentage (4.5%) of low HDL-c patients. Mutations in ABCA1 were found in all cases that exhibited impaired ABCA1 mediated efflux. This finding is in sharp contrast to the results of Mott et al, who found impaired cholesterol efflux (to apoA-I) from fibroblasts in 50% of the included 14 hypoalphalipoproteinemic patients. Further genetic analysis revealed that 29% (4/14) of those patients were carriers of an ABCA1 mutation. Based on these data, the authors suggested that a defect in cellular cholesterol efflux is a common cause of moderate to severe hypoalphalipoproteinemia (15).

The difference in prevalence of defects in ABCA1 mediated efflux and ABCA1 mutations in our low HDL-c patients could be a consequence of selection bias. Whereas Mott et al recruited their patients from the French Canadian population, we retrieved our patients from the general Dutch population. It has been well established that the prevalence of genetic variation depends on the selected population, and in this case the selection per se might be the cause for the observed differences. Moreover, Mott and colleagues considered a >40% reduction in ABCA1 mediated efflux as abnormal. We defined the lower limit at the mean efflux in 11 healthy, normolipidemic controls minus two times SD. Our lower limit equals an almost 50% reduction of the average of the 11 healthy controls (4.45/9.86x100 = 45%). Application of the 40% reduction limit of Mott, which would result in an absolute apoA-I value in our study of 5.9%, would only increase the number of patients to be sequenced by one and hence does not account for the much lower prevalence of efflux defects in our population.
Based on our results we might conclude that defects in ABCA1 are an infrequent cause for FHA in the Netherlands.

Our study does not allow an estimate of the true prevalence of ABCA1 mutations in the Dutch population. In a recent study, Wellington et al showed that cholesterol efflux in patients with one defective ABCA1 allele decreases significantly only when the mutation leads to a truncated protein (18). Apparently the presence of a truncated ABCA1 molecule negatively impacts on the activity of the intact protein. Thus, we might underestimate the prevalence of ABCA1 mutations in the general population, since some aminoacid substitutions may only be associated with a very mild phenotype.

Similar conclusions about the role of ABCA1 in FHA were recently described in a study focusing on the genetics, rather than on the activity of ABCA1. (19)

As we have previously reported, in the subset of patients with ABCA1 mutations we observed a strong correlation between efflux to apoA-I and serum HDL levels (9), indicating an important role for ABCA1 in these patients. This correlation could, however, not be generalized to the remainder of the cohort, in which efflux to apoA-I showed no correlation to serum HDL levels. Interestingly, efflux of cholesterol to HDL particles showed significant correlation to serum HDL. We hypothesize that efflux of cholesterol to lipid poor apoA-I is primarily controlled by ABCA1, whereas efflux to more mature HDL depends on other factors as well. In line with this is the observation that fibroblasts or macrophages derived from patients with Tangier disease show negligible efflux towards apoA-I, but efflux to HDL is reduced by only 50% in these patients. (7;8).

The nature of this non-ABCA1 dependent cholesterol efflux is not well understood. It may be due to passive diffusion of cholesterol, but unidentified proteins are likely to be involved in this process.
Low HDL-c levels were found to be hereditary, but our study cannot address the question whether the non-ABCA1 dependent efflux to HDL was inherited as well.

Assuming that the apoA-I dependent component in efflux to HDL is saturated, the apoA-I independent component may be calculated by subtracting the apoA-I mediated efflux from the HDL mediated efflux (Fig. 3) An alternative and perhaps better way to determine apoA-I independent efflux is to use methyl-β-cyclodextrin as an acceptor. In line with other studies (20;21) this compound induced a high rate of cholesterol efflux in an apparently aspecific way. Interestingly, also for this parameter a significant correlation between plasma HDL-c and efflux was observed (Fig. 4), which might suggest that non-specific, apoA-I independent efflux to mature HDL particles might importantly contribute to circulating HDL-c levels.

Our data therefore suggest that non-ABCA1 mediated cholesterol efflux is important for HDL-c levels in this group of patients and that other, to be discovered genetic defects might be responsible for the low levels of HDL-c found in these patients. Further research is required to identify these novel genes and to elucidate possible novel pathways involved in HDL metabolism.
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(6) Walter M, Gerdes U, Seedorf U, Assmann G. The high density lipoprotein- and apolipoprotein A-I-induced mobilization of cellular cholesterol is impaired in


LEGENDS

Figure 1. Lack of correlation between efflux of cholesterol to apoA-I and plasma HDL-cholesterol. Fibroblasts derived from 88 patients with FHA were loaded with $^3$H-cholesterol as described in Methods and efflux to 5µg/ml apo-AI was monitored. Data are expressed as percentage of total cellular cholesterol effluxed to apo-AI during a 20h incubation. Symbols: (▲) FHA patients, (○) patients with ABCA1 mutations (□) patients with ApoA-I mutations (◇) patient with LCAT mutation

Figure 2. Cholesterol efflux to HDL correlates with plasma HDL-cholesterol. Fibroblasts derived from 88 patients with FHA were loaded with $^3$H-cholesterol as described in Methods and efflux to 50µg/ml HDL was determined. Data are expressed as percentage of total cellular cholesterol effluxed to HDL during a 20h incubation. Symbols: (▲) FHA patients, (○) patients with ABCA1 mutations (□) patients with ApoA-I mutations (◇) patient with LCAT mutation

Figure 3. Relation between the apoA-I independent cholesterol efflux to HDL and plasma HDL-cholesterol concentration. Values for apoA-I independent cholesterol efflux to HDL were calculated but subtracting the values for efflux to apoA-I given in fig. 1 from the efflux to HDL given in fig. 2. Symbols: (▲) FHA patients, (○) patients with ABCA1 mutations (□) patients with ApoA-I mutations (◇) patient with LCAT mutation

Figure 4. Aspecific cholesterol efflux to methyl-β-cyclodextrin correlates with plasma HDL-cholesterol concentration. Fibroblasts derived from 88 patients with FHA were loaded with $^3$H-cholesterol as described in Methods and efflux to 1 mM methyl-β-cyclodextrin was measured. Data are expressed as percentage of total cellular cholesterol effluxed to methyl-β-
cyclodextrin during a 20h incubation. Symbols: (▲) FHA patients, (○) patients with ABCA1 mutations (□) patients with ApoA1 mutations (◇) patient with LCAT mutation
Fig. 1

HDL-C (mM) vs. Efflux to ApoA1 (%)
Fig. 2

Efflux to HDL (% of total)

HDL-C (mM)

$\rho = 0.34, p = 0.001$
Fig. 3

Efflux to HDL - efflux to Apo A1

Efflux to HDL-efflux to Apo A1

Serum HDL-C (mM)

Efflux to HDL-efflux to Apo A1

Efflux to HDL-efflux to Apo A1

Efflux to HDL-efflux to Apo A1

Efflux to HDL-efflux to Apo A1

r=0.32, p=0.008
Table 1: Baseline characteristics of 88 individuals with FHA

<table>
<thead>
<tr>
<th>Demographics</th>
<th>Value (±)</th>
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<tbody>
<tr>
<td>M / F</td>
<td>75 / 13</td>
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<tr>
<td>CAD / PVD n (percentage of total)</td>
<td>51 (58%)</td>
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<tr>
<td>Age (years)</td>
<td>53.09 (±11.81)</td>
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</table>

<table>
<thead>
<tr>
<th>Lipids and Lipoproteins</th>
<th>Value (±)</th>
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<tr>
<td>TC (mmol/L)</td>
<td>4.28 (±1.08)</td>
</tr>
<tr>
<td>LDL-c (mmol/L)</td>
<td>2.77 (±1.07)</td>
</tr>
<tr>
<td>HDL-c (mmol/L)</td>
<td>0.63 (±0.21)</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>1.83 (±1.07)</td>
</tr>
</tbody>
</table>

Values are given as means with standard deviation in parentheses. FHA = Familial Hypoalphalipoproteinemia, M / F = male / female ratio, CAD = Coronary Artery Disease, PVD = Peripheral Vascular Disease, TC = Total Cholesterol, LDL-c = low density lipoprotein cholesterol, HDL-c = high density lipoprotein cholesterol, TG = triglycerides.