Chapter 6

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Identification and characterization of novel loss of function mutations in ATP-binding cassette transporter A1 in patients with low plasma high density lipoprotein-cholesterol

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Abstract

Objectives The current literature provides little information on the frequency of mutations in the ATP binding cassette transporter A1 (ABCA1) gene in patients with low high-density lipoprotein cholesterol (HDL) levels that are referred to the clinic. In 78 patients with low plasma levels of HDL cholesterol that were referred to our clinic, we routinely screened for ABCA1 gene mutations and studied the functionality of newly identified ABCA1 missense mutations.

Methods. The coding regions and exon-intron boundaries of the ABCA1 gene were sequenced in 78 subjects with HDL cholesterol levels below the 10th percentile for age and gender. Novel mutations were studied by assessing cholesterol efflux capacity (using apolipoprotein A-I as acceptor) after transient expression of ABCA1 variants in BHK cells.

Results. Sixteen out of 78 patients (21%) were found to carry 19 different ABCA1 gene variants (1 frameshift, 2 splice-site, 4 nonsense and 12 missense variation) of which 14 variations were novel. Of three patients with homozygous mutations and three patients having compound heterozygous mutations only one patient presented with the clinical characteristics of Tangier Disease (TD) in the presence of nearly complete HDL deficiency. Seven out of 8 newly identified ABCA1 missense mutations were found to exhibit a statistically significant loss of cholesterol efflux capacity.

Conclusion. This study shows that 1 out of 5 patients who are referred to our hospital because of low HDL cholesterol levels have a functional ABCA1 gene mutation. It is furthermore demonstrated that in vitro studies are needed to assess functionality of ABCA1 missense mutations.
Introduction

Twin studies have indicated that the variation in plasma high-density lipoprotein (HDL) cholesterol levels is largely determined by genetic factors [1]. Many genes have been implicated in HDL metabolism [2] and this number is still expanding [3,4]. One of the major HDL candidate genes is the ATP-binding cassette transporter A1 (ABCA1) which is a cell membrane double transporter protein that plays an important role in cholesterol homeostasis. It is generally accepted that ABCA1 controls the rate-limiting step in the transport of cellular free cholesterol and phospholipids to apolipoprotein (apo) A-I which leads to the formation of pre-β high-density lipoprotein (pre-β HDL). Through the action of lecithin:cholesterol acyltransferase (LCAT), this pre-β HDL can mature into larger HDL subspecies [5-7].

Defects in the ABCA1 gene cause Tangier Disease (TD) [8], an autosomal recessive disorder characterized by HDL deficiency and accumulation of cholesterol in peripheral tissues. TD patients suffer from many symptoms including peripheral neuropathy, hepatosplenomegaly, and corneal opacification. While carriers of mutations in the ABCA1 gene are reported to exhibit an increased risk of atherosclerosis [9], it has also been reported that not all TD patients suffer from overt atherosclerosis [10]. Recent epidemiological data suggest that loss of ABCA1 function is not necessarily associated with increased risk of ischemic heart disease or cerebrovascular disease [11,12]. Heterozygous carriers for detrimental ABCA1 mutations do not present with specific clinical symptoms but present with markedly lower HDL cholesterol levels compared to age- and gender-matched controls [13].

ABCA1 is mainly expressed in the small intestine, liver, brain and cells of reticuloendothelial system. The 220 kDa protein is synthesized in the endoplasmic reticulum and transported to the plasma membrane via vesicles, but it is also found in intracellular compartments such as late endosomes/lysosomes, the trans-Golgi network and endoplasmic reticulum [6,14].

More than 50% of the over 90 identified ABCA1 mutations in the current literature [15] are missense mutations. Most of these mutations appear to be localized in extracellular loops, nuclear binding domains and carboxy terminal region [12,16,17]. Functionally defective ABCA1 variants fail to mediate lipid efflux to apo A-I and as a consequence the non-lipidated apo A-I fails to undergo maturation into larger HDL subspecies and will undergo rapid renal clearance [18]. Previous studies have demonstrated that missense mutations in ABCA1, identified in patients with Tangier disease or individuals with Familial hypoalphalipoproteinemia, can cause different degrees of impairment in lipid transfer activity [13].

Thus far, only few investigators have routinely sequenced the ABCA1 gene. There is only one report on patients with isolated low HDL cholesterol that are referred to the clinic [19]. Two other groups have reported ABCA1 gene variation at the lower end of the HDL cholesterol distribution curve of prospective epidemiological studies [11,17].
the current study, we sequenced the ABCA1 gene in 78 patients that were referred to our clinic who presented with HDL cholesterol levels below the 10th percentile for age and gender. We identified an unexpected high number of ABCA gene variants (n=19), of which 14 had not been described earlier. In vitro as well as confocal imaging experiments were carried out to evaluate whether the newly identified mutations were functional and could therefore explain the low HDL cholesterol phenotype of the respective patients.

Materials and methods

Study population
The current study is part of a research effort aiming at the characterization of mutations in established and newly proposed HDL genes, and the identification of novel genes that regulate HDL cholesterol levels. In a first step, we have selected 78 individuals with extremely low HDL cholesterol (<10th percentile for age and gender). In a second step, we have sequenced the coding regions of established HDL genes, i.e. ATP binding cassette transporter A1 (ABCA1), apolipoproteins A-I (apo A-I), and lecithin:cholesterol acyltransferase (LCAT). Patients were either seen in our outpatient clinic or they were referred to our hospital. With the exception of 5 patients from south-east Asia, 1 patient from Belgium and one patient from Spain, all other patients were of Dutch ancestry. Of note, patients #2 and #14 (see table 1) were referred with a suspicion of Tangier Disease. In this screening effort, we identified one mutation in APOAI in two subjects, 13 mutations in LCAT in 20 subjects and 19 mutations in ABCA1 in 16 individuals.

Biochemical measurements
Blood was obtained after an overnight fast in EDTA-coated tubes and directly placed on ice. Plasma was isolated by centrifugation at 4°C, 3000g for 15 minutes and stored at -80°C for further analyses. Plasma cholesterol, LDL cholesterol, HDL cholesterol and triglyceride levels were analyzed using a commercially available enzymatic method (Randox, Westburg, USA) on the Cobas Mira autoanalyzer (Roche, Basel, Switzerland).

Mutation screening in ABCA1
Genomic DNA was extracted from 10 ml whole blood on an AutopureLS apparatus according to manufacturer’s protocol (Gentra Systems, Minneapolis, USA). Primers were designed to amplify coding sequence and exon-intron boundaries of the ABCA1 gene using web-based Primer3 software [20]. PCR amplification was carried out with 50ng of genomic DNA in a 25μl reaction volume containing 1x Taq DNA polymerase buffer (Qiagen, Hilden, Germany), 50μmol/l of each dNTP, 0.4μmol/l of each primer, and 1U Taq DNA polymerase. A Touchdown PCR program (96°C for 5 minutes, then 20 cycles of 30 seconds at 96°C, 30 seconds at 65°C to 55°C with 0.5°C decrement/cycle and 30 seconds at 72°C, followed by 30 cycles of 30 seconds at 96°C, 30 seconds at 55°C and 30 seconds at 72°C) on a T3 biocycler PCR apparatus (Biometra, Germany) was used for
ABCA1 mutations in patients with low HDL-cholesterol

DNA amplification. The sequence reactions were performed using fluorescently labelled dideoxy chain terminations with a BigDye terminator ABI prism kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's protocol and analyzed on an Applied Biosystems automated DNA sequencer (model 370). Sequences were analyzed with the Sequencher Package (Gene Codes Co, Ann Arbor, Mi, USA).

Generation of ABCA1 gene expression vectors
Wild-type ABCA1-GFP pcDNA3.1 vector was provided by Prof. S. Calandra (University of Modena, Italy). This vector carries the human cDNA of ABCA1 fused in frame with a Green Fluorescence Protein (GFP) cDNA. 8 novel missense variations [c.299C>G (p.S100C), c.1724A>G (p.D575G), c.1779C>G (p.F593L), c.3167T>C (p.L1056P), c.3757G>A (p.E1253K), c.4535C>T (p.T1512M), c.5573T>C (p.V1858A), c.5821T>C (p.C1941R)] were introduced into this chimeric construct by site-directed mutagenesis using Stratagene QuikChange XL site-directed mutagenesis kit according to manufacturer's instructions (La Jolla, CA, USA).

Functional assessment of ABCA1 gene mutations
Baby hamster kidney (BHK) cells were obtained from ATCC (Manassas, VA). Cells were cultured in DMEM F-12 GlutaMax (GIBCO) containing 10% fetal bovine serum (FBS) and Penicillin (100U/ml)-Streptomycin (100ug/ml) at 37°C in a humidified 5% CO2 incubator. Transient transfections were carried out when the cells were at 90% confluency using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. Two days after transfection, transfection efficiency was evaluated by FACS analysis. The cells were harvested, centrifuged, extensively washed with phosphate buffered saline, resuspended in the same buffer and analyzed in a FacsCalibur cell sorter (BD Biosciences, Bedford, MA) using CellQuestPro software. Non-transfected cells were used as negative control. To assess cholesterol efflux potential, the transfected cells were incubated with 2μCi/ml [3H]cholesterol for 24 hours. Cholesterol efflux was measured after 4 h incubation with or without apoA-I (20μg/ml; Calbiochem). Radioactivity in the medium and cells was determined by scintillation counting and the fractional cholesterol efflux was calculated as the percentage of cpmmedium/(cpmmedium+cpmcell). For each construct, efflux to apo A-I was measured in triplicate in 3 independent experiments.

Confocal microscopy
BHK cells were plated on coverslips in 12-well plates at 30% confluency and transiently transfected with wild-type and mutant vectors after 24 hours. Cells were fixed with Methanol-Acetone 48 hours after transfection and mounted on slide using VECTASHIELD® Mounting Medium (VECTOR laboratories). Images were prepared using a Leica TCS-SP2 Confocal Microscope at 40x magnification and 488 nm wavelength.
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**Statistical analysis**

Statistical analyses were performed in SPSS version 16. Efflux data were analyzed using unpaired Student’s T-test statistics. Efflux data are presented as mean ± SD and p-values <0.05 were considered statistically significant.

**Results**

**ABCA1 gene defects in low HDL-c individuals**

In 78 patients with HDL cholesterol levels below the 10th percentile for age and gender, who were referred to our clinic, the coding sequence and exon-intron boundaries of the APOA1, LCAT and ABCA1 genes were sequenced. We identified 2 carriers of the APOAIp.L202P mutation, and 20 carriers of 13 different mutations in LCAT (data not shown). In ABCA1, we identified 14 novel and 5 known genetic variations in 16 subjects including one frameshift (p.C978fsX988), two splice site (IVS11-1G>C and IVS48+2T>C), 4 nonsense (p.R282X, p.W424X, p.Q1038X, p.W1747X) and 12 missense variations (p.S100C, p.D575G, p.F593L, p.L1056P, p.E1172D, p.S1181F, p.E1253K, p.C1477R, p.T1512M, p.N1800H, p.V1858A, p.C1941R). None of the 78 patients carried mutations in more than one of the 3 genes that were sequenced. The identified ABCA1 variations as well as the lipid profile and demographic data of the carriers are listed in Table 1. Seven ABCA1 variants were

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**Table 1: Identification of the ABCA1 mutation and baseline characteristics of the carriers (cDNA NM_005502).**

<table>
<thead>
<tr>
<th>Patients (gender, age)</th>
<th>Amino acid (Nucleotide) change</th>
<th>TC (mmol/l)</th>
<th>TG (mmol/l)</th>
<th>LDL-c (mmol/l)</th>
<th>HDL-c (mmol/l)</th>
<th>Clinical manifestations</th>
<th>CVD</th>
<th>Other relevant clinical data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1 (female, 42)</td>
<td>p.L1056P (c.3167T&gt;C)</td>
<td>2.4</td>
<td>0.9</td>
<td>1.99</td>
<td>&lt;0.05</td>
<td>Absent</td>
<td>CAD</td>
<td>None</td>
</tr>
<tr>
<td>Patient 2 (male, 50)</td>
<td>p.W747X (c.2240A&gt;G)</td>
<td>1.75</td>
<td>1.90</td>
<td>0.52</td>
<td>0.13</td>
<td>Neuropathy, Splenomegaly</td>
<td>CAD</td>
<td>None</td>
</tr>
<tr>
<td>Patient 3 (male, 65)</td>
<td>p.F593L (c.1779C&gt;T)</td>
<td>0.44</td>
<td>1.4</td>
<td>3.6</td>
<td>&lt;0.05</td>
<td>Absent</td>
<td>CAD</td>
<td>None</td>
</tr>
<tr>
<td>Compound heterozygotes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 4 (male, 50)</td>
<td>p.C038X (c.913G&gt;A)</td>
<td>0.05</td>
<td>2.72</td>
<td>5.4</td>
<td>&lt;0.05</td>
<td>Absent</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Patient 5 (male, 50)</td>
<td>p.H972Q (c.2916G&gt;C)</td>
<td>1.42</td>
<td>1.65</td>
<td>3.0</td>
<td>0.1</td>
<td>Absent</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Patient 6 (male, 26)</td>
<td>p.B116G (c.349A&gt;G)</td>
<td>2.90</td>
<td>2.64</td>
<td>0.37</td>
<td>&lt;0.05</td>
<td>Absent</td>
<td>CAD</td>
<td>None</td>
</tr>
<tr>
<td>Heterozygotes</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 7 (male, 40)</td>
<td>p.G129G (c.387G&gt;G)</td>
<td>0.5</td>
<td>1.3</td>
<td>4.1</td>
<td>0.9</td>
<td>N.A.</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Patient 8 (male, 50)</td>
<td>p.C1172G (c.3516G&gt;G)</td>
<td>1.6</td>
<td>1.7</td>
<td>4.1</td>
<td>0.9</td>
<td>N.A.</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Patient 9 (male, 50)</td>
<td>p.C978fs (c.2935delC)</td>
<td>2.9</td>
<td>0.5</td>
<td>0.9</td>
<td>0.9</td>
<td>N.A.</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Patient 10 (male, 45)</td>
<td>p.C1767R (c.5292G&gt;T)</td>
<td>3.01</td>
<td>1.4</td>
<td>0.92</td>
<td>0.46</td>
<td>N.A.</td>
<td>CAD</td>
<td>None</td>
</tr>
<tr>
<td>Patient 11 (male, 50)</td>
<td>p.K1344R (c.4033C&gt;T)</td>
<td>4.9</td>
<td>3.78</td>
<td>2.4</td>
<td>0.75</td>
<td>N.A.</td>
<td>CAD</td>
<td>None</td>
</tr>
<tr>
<td>Patient 12 (male, 36)</td>
<td>p.L1056P (c.3167T&gt;C)</td>
<td>0.44</td>
<td>1.2</td>
<td>3.0</td>
<td>&lt;0.13</td>
<td>N.A.</td>
<td>None</td>
<td>DM2</td>
</tr>
<tr>
<td>Patient 13 (male, 50)</td>
<td>p.R282X (c.846G&gt;T)</td>
<td>0.2</td>
<td>1.21</td>
<td>2.14</td>
<td>0.51</td>
<td>N.A.</td>
<td>None</td>
<td>DM2</td>
</tr>
<tr>
<td>Patient 14 (male, 42)</td>
<td>p.W424X (c.1273C&gt;A)</td>
<td>2.07</td>
<td>1.54</td>
<td>1.38</td>
<td>0.21</td>
<td>N.A.</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Patient 15 (male, 50)</td>
<td>p.S1181F (c.3543C&gt;G)</td>
<td>0.54</td>
<td>1.32</td>
<td>3.84</td>
<td>0.49</td>
<td>N.A.</td>
<td>None</td>
<td>DM2</td>
</tr>
<tr>
<td>Patient 16 (male, 50)</td>
<td>p.E1172D (c.3516G&gt;G)</td>
<td>1.42</td>
<td>1.32</td>
<td>3.84</td>
<td>0.49</td>
<td>N.A.</td>
<td>None</td>
<td>DM2</td>
</tr>
</tbody>
</table>

*Nomenclature based on guidelines of Human Genome Variation Society. Plasma Lipid values are presented as mmol/l. TC: Total cholesterol, TG: Triglyceride, CVD: Cardiovascular disease, TD: Tangier Disease, N.A.: Not Applicable, DM1: Diabetes Mellitus type 1, DM2: Diabetes Mellitus type 2.
ABCA1 mutations in patients with low HDL-cholesterol

considered to be functional due to obvious destructive effects of frameshift, splice site and nonsense variations on protein structure and/or function. In addition, 5 missense variations have previously been reported to be functional (references are given in Table 1) and were not analyzed in the current study.

From 8 novel missense variations identified in our cohort, one is localized in the first transmembrane domain (p.S100C), two in the first large extracellular loop (p.D575G and p.F593L), two in the first Nuclear Binding Domain (p.L1056P and p.E1253K), one in the second large extracellular loop (p.T1512M), one in the extracellular region, close to the plasma membrane (p.V1858A) and one is localized in the C-terminal domain (p.C1941R). The estimated positions of these variants are given in Figure 1.

In silico prediction of the effect of ABCA1 variations

In silico analysis was performed using PolyPhen (=Polyorphism Phenotyping) [21] and SIFT [22] software to predict functional significance of the 8 novel missense variations (see supplementary table 1). Four out of 8 mutations were predicted to be probably damaging (p.S100C, p.D575G, p.T1512M, p.C1941R), two as possibly damaging (p.F593L and p.L1056P) and two were described as benign (p.E1253K and p.V1858A) by PolyPhen. Using SIFT, 7 novel variations were predicted to affect protein function, while one (p.V1858A) was predicted to be tolerated.

Figure 1: Position of the newly identified ABCA1 variations in ABCA1 protein structure.
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*In vitro characterization of novel missense ABCA1 variations in BHK cell system*

All newly identified missense variations were studied *in vitro* by assessing the ABCA1-mediated cholesterol efflux to apo A-I in BHK cells that were transiently transfected with wild-type or mutant ABCA1 constructs. FACS analysis of transfected cells (using GFP-tagged ABCA1 protein) indicated that all ABCA1 variants were expressed at comparable levels to that of the wild-type protein (data not shown).

Efflux data of three independent triplicate experiments were normalized to the values from the cells transfected with the wild-type ABCA1 construct. Figure 2 shows that the ABCA1- p.S100C, p.D575G, p.F593L, p.L1056P, p.E1253K, p.T1512M, p.C1941R mutant proteins all had a significantly reduced capacity to efflux cholesterol to apo A-I compared to wild-type ABCA1 which is in line with the low HDL cholesterol levels of the individuals in whom the mutations were identified. The ABCA1- p.V1858A, however, had a normal potential to efflux cholesterol to apo A-I which is in line with the prediction that the mutation is benign (both PolyPhen and SIFT) suggesting that the low HDL cholesterol in this individual is likely the result of other, yet unknown molecular defect.

**Figure 2:** Normalized cholesterol efflux to ApoA-I in BHK cells transfected with ABCA1 variants relative to cells transfected with wild-type ABCA1 (WT).

<table>
<thead>
<tr>
<th>ABCA1 variants</th>
<th>% Efflux relative to WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT: non-transfected cells.</td>
<td>* p&lt;0.05, ** p&lt;0.01, *** p&lt;0.001</td>
</tr>
<tr>
<td>WT</td>
<td>100</td>
</tr>
<tr>
<td>p.S100C</td>
<td>30*</td>
</tr>
<tr>
<td>p.D575G</td>
<td>50**</td>
</tr>
<tr>
<td>p.F593L</td>
<td>70***</td>
</tr>
<tr>
<td>p.L1056P</td>
<td>90***</td>
</tr>
<tr>
<td>p.E1253K</td>
<td>110***</td>
</tr>
<tr>
<td>p.T1512M</td>
<td>130***</td>
</tr>
<tr>
<td>p.C1941R</td>
<td>150***</td>
</tr>
<tr>
<td>p.V1858A</td>
<td>100</td>
</tr>
</tbody>
</table>

**Cellular localization of mutant ABCA1 proteins**

GFP-tagged wild-type and novel mutant proteins were expressed in BHK cells and their localization was evaluated using confocal microscopy. While WT-ABCA1 and ABCA1-V1858A variant show normal localization on plasma membrane, ABCA1-L1056 protein shows a complete intracellular retention. All other mutant ABCA1 proteins show partial intracellular retention (Supplementary figure 1).

**Discussion**

Our group has a long-standing interest in the characterization of the genetic background of low HDL cholesterol levels of patients that are referred to our hospital. In the current
paper, we focus on the identification and function of newly identified ABCA1 gene mutations.

**Frequency of ABCA1 gene mutations**

Routine screening for ABCA1 mutations in 78 patients with HDL cholesterol levels below the 10th percentile revealed 5 known and 14 novel gene variations. An approximate 21% mutation rate suggests a high prevalence of ABCA1 mutations in patients with low HDL-c that are referred to our institute. A similar sequencing effort in patients that were referred to the hospital was carried out by Kiss et al [19] who identified a much lower prevalence of ABCA1 mutations of 6%. We have no clear explanation for the discrepancy other than that Kiss et al. have used less strict inclusion criteria (including diabetes, short bowel syndrome, nephrotic syndrome, malignancies including multiple myeloma, or treatment with stanozolol or danazol). Instead, we have solely selected patients on the basis of low HDL cholesterol levels. We can moreover not exclude a referral bias for inherited HDL traits, since our group has a long history in the characterization of genetic disorders of HDL metabolism. In fact, 2 of 78 patients were referred due to TD symptoms. The ABCA1 gene has also been sequenced in individuals at the lower end of the HDL cholesterol distribution curve of prospective epidemiological studies [11,17]. The observed frequencies were also lower, i.e. 10.9 % in the Dallas Heart Study (HDL cholesterol < 5th percentile; HDL cholesterol <0.76 mmol/l for men and <0.81 mmol/l for women) and 10% in the Copenhagen City Heart Study (bottom 1st percentile; HDL cholesterol <0.6 mmol/l for men and <0.8 mmol/l for women). Several investigators have identified ABCA1 promoter polymorphisms that are associated with HDL cholesterol levels [11,23] although only one study shows functionality of a mutation in the promoter region [11]. In the present study, we did not sequence the regulatory sequences of the ABCA1 gene, indicating that we may underestimate the frequency of ABCA1 mutations in our cohort.

**Impact of novel missense mutations on ABCA1 function**

The newly identified missense mutations were further studied in *in silico* and *in vitro* experiments which allowed for head-to-head comparisons between the outcomes. Nowadays, *in silico* prediction of the functionality of mutations has become very popular [17] mainly due to its simplicity and ease of use in the context of the magnitude of genetic data that are generated by second generation sequencing technology. It has previously been shown that these methods have their limitations [24]. In line, the current study shows that predictions made by PolyPhen were consistent with *in vitro* characterization studies in only 50% of the cases (Supplementary table 1). The most striking discrepancy was found for the new ABCA1-p.L1056P variant which was only defined as possibly damaging while our data show that this variant is amongst those with the most profound loss of ABCA1 mediated efflux and confocal microscopy revealed complete intracellular retention (Supplementary figure 1). In line, cholesterol efflux of fibroblasts grown from
skin biopsies of the index patient who was homozygous for this mutation (a classical test for diagnosis of TD) indicated complete abrogation of cholesterol efflux to apo A-I as an acceptor (Supplementary figure 2).

In contrast to PolyPhen, SIFT predictions for the assessment of the novel variations studied here were more compatible to the outcome of our in vitro experiments, i.e. all variants with significant reduction in cholesterol efflux to apo A-I in our in vitro assay were proposed to be deleterious while ABCA1-p.V1858A was predicted to be tolerated.

Combined, the current data indicate the importance of assessing the effect of missense mutations through functional studies and to not solely rely on in silico predictions. Many investigators have shown that ABCA1 mutations can have an impact on the 3-dimensional folding of the protein. The position of the mutation can affect maturation, localization or interaction of ABCA1 with early/late endosomes, lysosomes, phospholipids of the plasma membrane. More importantly it may interfere with its capacity to interact with apo A-I [25-27] and thus lead to a decrease in the synthesis of pre-β HDL. The missense mutations ABCA1-p.L1056P and ABCA1-p.E1253K, identified in patients with near HDL deficiency, are located in the intracellular region, inside the nuclear binding domain 1, close to the Walker A motif and after the Walker B motif respectively (Figure 1). This region is known to be involved in the ATP hydrolysis function of ABCA1 [28,29]. Mutations located in this region may have an impact on the ability of ABCA1 to hydrolyze ATP which is necessary to transport its substrates [30]. Thus, lack of proper localization to the plasma membrane for ABCA1-p.L1056P and partial intracellular retention for ABCA1-p.E1253K (Supplementary figure 1) results in low cholesterol efflux potential of both ABCA1 mutants and confirms the vital role of this ABCA1 domain (Figure 2).

Also, mutations in extracellular loops have been shown to affect the protein’s folding, localization, lipid transport ability [29,31] or apo A-I binding [6]. The ABCA1- p.F593L and ABCA1-p.D575G mutations are located in the first large extracellular loop, while ABCA1-p.T1512M is located in the second extracellular loop. All 3 mutant proteins are indeed hampered in their ability to efflux cholesterol to apo A-I (Figure 2). In line, the mutant proteins are retained into the intracellular compartments and have a limited expression at the cell membrane (Supplementary figure 1). All mutations were identified in patients with almost complete HDL deficiency.

The cholesterol efflux assay performed for ABCA1-p.S100C revealed a significant reduction in efflux potential to apo A-I. The mutation is located in the first transmembrane domain and may impair the protein’s ability to properly interact with the plasma membrane. The ABCA1-p.C1941R mutation showed a marked reduction in cholesterol efflux. This mutation is localized in the C-terminal domain which may lead to defective localization or oligomerization [32], especially due to proximity to the PDZ binding domain. Confocal microscopy also revealed considerable intracellular retention of this mutant (Supplementary figure 1).
The ABCA1-p.V1858A variant was the only missense variation that was found to have no significant effect on cholesterol efflux and cellular localization. It is possible that the extracellular region, where the variant is located, is not fundamental for ABCA1 folding and function.

In conclusion, the current study shows that ABCA1 gene defects explain up to 20% of the low HDL cholesterol phenotype of patients that are referred to our clinic. In view of the scarcity of routine ABCA1 gene sequencing efforts in referred patients, further studies are needed to verify partial ABCA1 deficiency as a frequent cause of low HDL cholesterol levels. Through functional studies we furthermore showed that the majority of the newly identified missense mutations were functional.

Acknowledgements

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Supplementary Figure 1: Localization of GFP-tagged ABCA1 WT and GFP-tagged ABCA1 mutant proteins in BHK cells. (for color figure, see page 417)

Supplementary Table 1: Relative percentage of apo A-I-mediated cholesterol efflux using BHK cells transfected with ABCA1 variants compared to the wild-type protein.

<table>
<thead>
<tr>
<th>ABCA1 Variant</th>
<th>% efflux relative to WT (Mean±SD)</th>
<th>p-value</th>
<th>SIFT prediction</th>
<th>PolyPhen prediction</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT</td>
<td>10.4 ± 1.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p.L1056P</td>
<td>36.4 ± 2.7</td>
<td>&lt;0.0001</td>
<td>Affects protein function</td>
<td>Possibly damaging</td>
</tr>
<tr>
<td>p.F593L</td>
<td>70.9 ± 5.6</td>
<td>0.0006</td>
<td>Affects protein function</td>
<td>Possibly damaging</td>
</tr>
<tr>
<td>p.E1253K</td>
<td>81.6 ± 6.3</td>
<td>0.0085</td>
<td>Affects protein function</td>
<td>Benign</td>
</tr>
<tr>
<td>p.T1512M</td>
<td>87.8 ± 4.8</td>
<td>0.0244</td>
<td>Affects protein function</td>
<td>Probably damaging</td>
</tr>
<tr>
<td>p.D575G</td>
<td>60.9 ± 9.3</td>
<td>0.0022</td>
<td>Affects protein function</td>
<td>Probably damaging</td>
</tr>
<tr>
<td>p.C1941R</td>
<td>49.8 ± 6.4</td>
<td>0.0002</td>
<td>Affects protein function</td>
<td>Probably damaging</td>
</tr>
<tr>
<td>p.S100C</td>
<td>75.6 ± 3.4</td>
<td>0.0004</td>
<td>Affects protein function</td>
<td>Probably damaging</td>
</tr>
<tr>
<td>p.V1858A</td>
<td>89.1 ± 5.2</td>
<td>0.0831</td>
<td>Tolerated</td>
<td>Benign</td>
</tr>
</tbody>
</table>

NT: Non-transfected cells. Analysis is performed using unpaired Student’s T-test statistics.
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Absence of cholesterol efflux to apo A-I could clearly be seen in monocytes isolated from a patient with homozygote p.L1056P mutation (L1056P-/-). Monocytes from a parent (L1056P+/-) showed reduced efflux to apoA-I (<50% of efflux values in control monocytes). **Experimental setup:** 30 ml of heparinised blood was collected and diluted 1:1 with PBS/0.1% bovine serum albumin (BSA) + heparin (LeoPharma). To isolate the mononuclear cells, this mixture was layered on Lymphoprep 1.077g/ml (Lucron Bioproducts N1114544) and centrifuged for 15 minutes at 1000g. The interface was collected and washed twice. Thrombocytes were subsequently depleted by centrifugation for 10 minutes at 250g without brake. In a next step, the mononuclear cells were layered on a Percoll (Pharmacia 17-0891-01) density gradient and centrifuged for 45 minutes at 1750g. The monocytes were harvested from the upper interface and washed twice. 0.4 x 10⁶ cells were seeded in 1 ml of RPMI 1640/glutamax/hepes (Gibco 72400) with 10% human serum, in a 24-wells cell culture plate. Cells were cultured for 10 days. Cells were then loaded overnight at 37°C with free [3H]cholesterol, by adding 0.5ml of labeling medium (RPMI, Glutamax, 0.1% Pen/Strep, 0.2% BSA, 30μg/ml cholesterol, 0.5μCi/ml [3H]cholesterol in ethanol) in the presence of 3mM LXR agonist TO-901317 (#71810, Cayman Chemical Company). Labeling medium was removed and the cells were washed four times, after which 0.5ml of efflux medium, consisting of RPMI, Glutamax, 0.1% P/S, 0.2% BSA and 10μg/ml human apoA-I (Calbiochem) or native human HDL 12ug/ml (Calbiochem) was added to each well. Efflux medium without apoA-I or HDL was used as control. Efflux was measured over a 4-hour time span.

**Supplementary Figure 2:** Monocyte cholesterol efflux to apo A-I in patient-derived monocyte-macrophages relative to monocyte-macrophages obtained from healthy volunteers.