Identification and targeting of genes in atherosclerosis
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Chapter 4

Two novel mutations in Apolipoprotein C3 underlie atheroprotective lipid profiles in families


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ABSTRACT

Objectives: Apolipoprotein C3 (APOC3) mutations carriers typically display high plasma high-density lipoprotein cholesterol (HDL-C) and low triglycerides. We set out to investigate the prevalence and clinical consequences of APOC3 mutations in individuals with hyperalphalipoproteinemia.

Methods and results: Two novel mutations (c.-13-2A>G and c.55+1G>A) and one known mutation (c.127G>A;p.Ala43Thr) were found. Lipid profiles and apoCIII isoform distributions were measured. c.55+1G>A mutation carriers displayed higher HDL-C percentiles (35.6±35.8 vs 99.0±0, p=0.002) and lower triglycerides (0.51 (0.37-0.61) vs 1.42 (1.12-1.81) mmol/L, p=0.007) and apoCIII levels (4.24±1.57 vs 7.33±3.61 mg/dL, p=0.18). c.-13-2A>G mutation carriers did not display significantly different HDL-C levels (84.0±30.0 vs 63.7±45.7, p=0.50), a trend towards lower triglycerides (0.71 (0.54-0.78) vs 0.85 (0.85-~) mmol/L, p=0.06) and significantly lower apoCIII levels (3.09±1.08 vs 11.45±1.06 mg/dL, p=0.003). p.Ala43Thr mutation carriers displayed a trend towards higher HDL-C percentiles (91.2±31.8 vs 41.0±29.7 mmol/L, p=0.06) and significantly lower triglycerides (0.58 (0.36-0.63) vs 0.95 (0.71-1.20) mmol/L, p=0.02) and apoCIII levels (4.92±2.33 vs 6.60±1.60, p=0.25).

Conclusion: Heterozygosity for APOC3 mutations results in high HDL-C and low triglycerides and apoCIII levels. This favourable lipid profile in patients with genetically low apoCIII levels holds promise for current studies investigating the potential of apoCIII inhibition as a novel therapeutic in CVD prevention.
Identification of two novel APOC3 mutations

INTRODUCTION

Large prospective epidemiological studies have consistently demonstrated a strong inverse relationship between plasma levels of high-density lipoprotein cholesterol (HDL-C) and the risk of cardiovascular disease (CVD).\textsuperscript{1-3} Furthermore, fasting\textsuperscript{4} as well as postprandial\textsuperscript{5,6} triglyceride (TG) levels have been shown to be associated with CVD risk. Plasma HDL-C and triglyceride levels are mechanistically closely and inversely linked. Apolipoprotein (apo) CIII plays a pivotal role in HDL-C and TG metabolism, by virtue of its lipoprotein lipase (LPL) inhibiting activity. In genome wide association studies (GWAS), genetic variants in \textit{APOC3} are both associated with high HDL-C levels as well as low triglyceride levels.\textsuperscript{7} Moreover, common variations in \textit{APOC3} have been shown to be associated with CVD risk.\textsuperscript{8} Pollin et al confirmed the importance of apoCIII as an independent risk factor for CVD in humans by showing that carriers of a null mutation in \textit{APOC3} exhibit 50\% of normal plasma apoCIII levels, lower fasting and postprandial serum triglycerides and less subclinical atherosclerosis, as measured by the extent of coronary artery calcification.\textsuperscript{9}

Studies in patients with familial disorders of lipoprotein metabolism have led to significant advances in our understanding of HDL metabolism, including the identification of key players involved in HDL biogenesis, transport and modification such as ATP binding cassette transporter Al (\textit{ABCA1}),\textsuperscript{10} apolipoprotein Al (\textit{APOA1}),\textsuperscript{11} lecithin cholesterol acyl transferase (\textit{LCAT})\textsuperscript{12,13} and cholesteryl ester transfer protein (\textit{CETP}).\textsuperscript{14} ApoA1, LCAT and CETP-based therapies could indeed modify lipid profiles in a favourable manner, especially in terms of HDL-C levels. However, none of these therapies have thus far shown a consistent beneficial effect on CVD outcome or atherosclerosis progression.\textsuperscript{15-19} Whereas increasing plasma HDL-C by CETP inhibition does not lead to the desired CVD risk reduction,\textsuperscript{15-17} fibrates, both lowering triglycerides and increasing HDL-C, reduce CVD risk in subgroups characterized by high triglycerides.\textsuperscript{20} Noteworthy, apoCIII plasma levels are inversely associated with both plasma triglyceride levels and CVD risk. This indicates that in large populations, inhibition of apoCIII may be beneficial. In line with this, antisense inhibition of apoCIII induced reductions of plasma apoCIII and triglycerides in a recent phase I study.\textsuperscript{21} A phase II antisense intervention trial (ISIS-APOCIII\textsubscript{Rx}. Clinical Trials. gov: NCT01529424) has been started.

We set out to study the consequences of novel mutations in \textit{APOC3} in an independent cohort. In this paper, we report the lipid profiles as well as apoCIII isoform distribution in carriers of two new \textit{APOC3} mutations and one previously published mutation.
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MATERIALS AND METHODS

Recruitment of the Study Family

The coding regions of APOC3 were sequenced in 80 unrelated probands with plasma HDL-C > 95th percentile for age and gender. Family members of APOC3 mutation carriers were recruited. Unaffected family members participated as controls. All participants provided written informed consent. The study was conducted at the Academic Medical Center in Amsterdam, the Netherlands. The study protocol was approved by the Institutional Review Board of the Academic Medical Center in Amsterdam, the Netherlands.

Mutation analysis

Genomic DNA was extracted from 10 ml whole blood on an Autopure LS system according to the manufacturer’s instructions (Gentra Systems, Minneapolis, USA). APOC3 was sequenced using filter-based hybridization capture as described. Mutations were validated using conventional Sanger sequencing. Primers were designed using Primer 3 software to cover the identified mutations in APOC3 (NM_000041.1). An M13 tail was added to each primer (forward: 5’-GTTGTAAAACGACGGCCACT-3’ and reverse: 5’-CACAGGAAACAGCTATGACC-3’) to facilitate DNA sequencing. Sequence reactions were performed as described. The coding DNA numbering starts at the ATG transcription start site according to the official nomenclature provided by the Human Genome Variation Society (www.hgvs.org/mutnomen).

Patient characteristics

Presence of cardiovascular risk factors, use of medication and family history of CVD were assessed by a questionnaire. Blood was obtained after an overnight fast and stored at -80 °C. Plasma cholesterol, low-density lipoprotein cholesterol (LDL-C), HDL-C and triglycerides were analysed using commercially available kits (Randox, Antrim, United Kingdom and Wako, Neuss, Germany). Since gender was (unequally distributed between carriers and family controls, HDL-C percentiles for age and gender are calculated. Plasma apoA-I, apoB and apoCIII (Randox, Antrim, United Kingdom) were measured using a commercially available turbidometric assay. All analyses were performed using the Cobas Mira autoanalyzer (Roche, Basel, Switzerland).

Fast performance liquid chromatography

Individual patient plasma samples were fractioned using fast performance liquid chromatography (FPLC) analysis to determine lipoprotein particle size. In brief, the system-
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contained a PU-980 ternary pump with an LG-980-02 linear degasser, a FP-920 fluorescence and UV-975 UV/VIS detector (Jasco, Tokyo, Japan). An extra P-50 pump (Pharmacia Biotech, Uppsala, Sweden) was used for in-line cholesterol and TG (4-Amino-antiperine-peroxidase) PAP enzymatic reagent (Biomerieux, Marcy l’Etoile, France) addition at 0.1 mL/min. Ethylenediaminetetraacetic acid (EDTA) plasma was diluted 1:1 with Tris buffered saline and 60 μL sample-buffer mixture was loaded on a Superose 6 HR 10/30 column (GE Health care, Life sciences division, Diegem, Belgium) for lipoprotein separation at a flow rate of 0.31 mL/min.

ApoCIII isoforms and distribution among lipoproteins

Plasma apoCIII isoforms were separated with isoelectric focusing (IEF) gel electrophoresis on the Phast-gel system (Pharmacia) as previously described. Two Phastgel Dry IEF gels were hydrated with 5 ml solution containing 8M urea, 390 μl pharmalyte 4.2-4.9; 195 μl pharmalyte 2.5-5.0 for 1.5h at room temperature. Plasma was diluted with 0.9% NaCl (15 times) followed by application on IEF gels. Samples were run on standard program 2 of the Phast system followed by transfer to polycyline polyvinylidene fluoride (PVDF) transfer membranes (Perkin Elmer NEF1002), blocked with Odyssey blocking buffer (Westburg) for 1 h and incubated with rabbit anti human apoCIII (Abcam 21032; 1:2000) o/n at room temperature and after extensive washing stained for 1h at room temperature with donkey anti rabbit IRdye 800CW (1:5000; Westburg, 926-32212). Blots were imaged on the Odyssey system (Licor, Westburg). IEF resolves apoCIII into three isoforms, apoCIII0, apoCIII1, and apoCIII2.

Distribution of apoCIII among HDL and TG-rich apoB-containing lipoprotein particles was analysed using immunoelectrophoresis using LpCIII hydragel (Sebia) according to the manufacturer’s manual.

Statistical analysis

Continuous variables are expressed as means ± standard deviations (SD), unless otherwise specified. Differences in demographic, biometrical and biochemical parameters between carriers of APOC3 mutations and controls were assessed using unpaired Student’s t-tests or Chi square tests, where appropriate. A p-value of 0.05 or less was considered statistically significant.
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RESULTS

APOC3 Mutations

In 5 of the 80 individuals (6%) we identified three mutations in APOC3 (NM_000040.1): Three heterozygous carriers of c.55+1G>A (rs138326449), one heterozygous carrier of c.-13-2A>G and one heterozygous carrier of c.127G>A (p.Ala43Thr, rs147210663). The p.Ala43Thr mutation was previously known as p.Ala23Thr. The new mutation nomenclature guidelines (http://www.dmd.nl/mutnomen/recs-prot.html) set the ATG start site as codon number 1. This explains the 20 amino acid difference compared to the old nomenclature. None of the mutations were found in 40 individuals with low HDL-C levels. The frequencies of these mutations in a population sample of 4000 individuals were very low (minor allele frequency of 0.0019, 0.0001 and 0.0009, respectively (exome variant server, NHLB1 GO Exome Sequencing Project, 2013). The c.55+1G>A (rs138326449) mutation leads to a change in a highly conserved nucleotide and disrupts the canonical splice site, leading to a predicted skipping of exon 2, which may affect the apoCIII protein. The p.Ala43Thr mutation is located in exon 3 and changes a highly conserved nucleotide leading to a change in amino acid 43. p.Ala43Thr is predicted to be deleterious by SIFT prediction software. The c.-13-2A>G mutation is located in intron 1, prior to the ATG start site in exon 2. This mutation is predicted to disrupt the splice site upstream of exon 2.

Pedigrees

Pedigrees A, B and C are shown in figure 1. Two of the c.55+1G>A mutation carriers were from families which could not be further expanded. Their pedigrees are not shown. Cosegregation of the variants with the lipid phenotype (high HDL-C/low triglycerides) was observed with the exception of one case of non-penetrance in pedigree C. There was one phenocopy in pedigree A.

Figure 1: pedigrees (on next page)

a. pedigree A: c.-13-2A>G
b. Pedigree B: C.55+1G>A
c. pedigree C: p.Ala43Thr

pTC is percentile of total cholesterol, pLDL is percentile of low-density lipoprotein cholesterol
pHDL is percentile of high-density lipoprotein cholesterol, pTG is percentile of triglycerides.
All values are given in mmol/L
Identification of two novel APOC3 mutations

a. Pedigree A: c.-13+2A>G

b. Pedigree B: c.554+1G>A

c. Pedigree C: p.Ala43Thr

Figure 1: pedigrees
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<table>
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<tr>
<th></th>
<th>Pedigree A</th>
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<td>4</td>
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<td>49±21</td>
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<td>22.7±2.5</td>
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<td>Alcohol intake (units per week)</td>
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<td>Medication use, n (%)</td>
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<td>0</td>
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<td>Asprin</td>
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<td>1 (13)</td>
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<td>Presence of diabetes, n (%)</td>
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<td>Lipid metabolism</td>
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<td>Total cholesterol (mmol/L)</td>
<td>4.77±0.12</td>
<td>5.15±0.94</td>
<td>5.49±0.74</td>
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<td>LDL-cholesterol (mmol/L)</td>
<td>2.58±0.48</td>
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<td>HDL-cholesterol (mmol/L)</td>
<td>1.81±0.71</td>
<td>1.80±0.24</td>
<td>1.16±0.26</td>
<td>2.25±0.41</td>
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<td>HDL-cholesterol percentile</td>
<td>63.7±45.7</td>
<td>84.0±30.0</td>
<td>35.6±35.8</td>
<td>99.0±0</td>
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<td>Triglycerides (mmol/L)</td>
<td>0.85 *</td>
<td>0.71 (0.54-0.78)</td>
<td>1.42 (1.12-1.81)</td>
<td>0.51 (0.37-0.61)</td>
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<tr>
<td>Apolipoprotein B (g/L)</td>
<td>0.91±0.22</td>
<td>1.04±0.35</td>
<td>0.98±0.00</td>
<td>0.92±0.46</td>
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<td>Apolipoprotein A-I (g/L)</td>
<td>1.92±0.02</td>
<td>1.78±0.12</td>
<td>1.14±0.22</td>
<td>1.81±0.21</td>
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<td>Apolipoprotein C3 (mg/dl)</td>
<td>11.45±1.06</td>
<td>3.09±1.08</td>
<td>7.33±3.61</td>
<td>4.24±1.57</td>
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<td>Apo-C3 in HDL (%)</td>
<td>74±11</td>
<td>90±13</td>
<td>71±8</td>
<td>78±27</td>
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<tr>
<td>Apo-C3 in non-HDL (%)</td>
<td>26±11</td>
<td>11±13</td>
<td>29±8</td>
<td>22±27</td>
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<tr>
<td>Particle size</td>
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<td>Retention time VLDL (minutes)</td>
<td>28.0±0.05</td>
<td>28.0±0.01</td>
<td>27.9±0.16</td>
<td>28.0±0.20</td>
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<tr>
<td>Retention time LDL (minutes)</td>
<td>35.4±0.6</td>
<td>35.8±0.3</td>
<td>35.3±0.4</td>
<td>35.5±0.4</td>
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<tr>
<td>Retention time HDL (minutes)</td>
<td>49.5±1.4</td>
<td>49.8±0.5</td>
<td>49.8±0.6</td>
<td>48.9±0.9</td>
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Table 1. Baseline characteristics Values are presented as mean ± SD unless otherwise indicated. Male sex, smokers, medication use, history of cardiovascular disease, presence of hypertension, presence of diabetes: p for χ2 test; other parameters: p for student’s t-test. alcohol intake and tg, median and interquartile range is reported; P for Mann Whitney U test.
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Baseline characteristics of APOC3 mutation carriers and family controls are shown in table 1. HDL-C levels in heterozygous carriers of p.Ala43Thr were not significantly different from controls (2.19±0.77 vs 1.43±0.32, p=0.10). However, HDL-C percentiles, correcting for the unequal gender distribution between carriers and family controls, showed a trend towards an increase (91.17±31.83 vs 41.00±29.68 mmol/L, p=0.06) whereas triglyceride levels were significantly lower (0.58 (0.36-0.63) vs 0.95 (0.71-1.20) mmol/L, p=0.02). There was no difference in HDL size between carriers and non-carriers (HDL peak: 50.07 ±0.43 vs 49.60 ±0.65 minutes retention time, p=0.26). HDL-C levels and HDL-C percentiles were higher in heterozygous c.55+1G>A mutation carriers compared to family controls (2.25±0.41 vs 1.16±0.26, p=<0.001 and 35.63±35.77 vs 99.00±0, p=0.002 respectively) and triglyceride levels were lower (0.51 (0.37-0.61) vs 1.42 (1.12-1.81) mmol/L, p=0.007). HDL size did not differ (48.88±0.86 vs 49.79±0.63 minutes retention time, p=0.18). In c.-13-2A>G mutation carriers, neither HDL-C levels (1.80±0.24 vs 1.81±0.71), nor HDL-C percentiles (35.4±0.4 vs 36±14, p=0.78) were significantly different, whereas carriers displayed a trend towards lower triglyceride levels (0.71 (0.54-0.78) vs 0.85 (0.85-~), p=0.06).
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Plasma apoCIII isoforms and distribution among lipoproteins

Plasma apoCIII levels were lower in all mutation carriers. This difference was significant for c.-13-2A>G (p=0.003), but did not reach significance for c.55+1G>A and p.Ala43Thr mutation carriers (p=0.18 and 0.25 respectively) (figure 2). None of the apoCIII isoform distributions differed from control values (figure 3). ApoCIII is present on HDL and TG-rich apoB-containing lipoproteins. The percentage of apoCIII in HDL did not differ for c.-13-2A>G (74±11 vs 90±13, p=0.17), c.55+1G>A (71 ±8 vs 78 ±28, p=0.69) or p.Ala43Thr (67±4 vs 64±13, p=0.78).

Figure 2. ApoCIII plasma levels

Figure 3. ApoCIII isoform distribution
DISCUSSION

We have identified two new mutations in *APOC3* (c.-13-2A>G and c.55+1G>A) and one previously identified mutation (c.127G>A; p.Ala43Thr). The mutations were found in five individuals with plasma HDL-C above the 95th percentile. The atheroprotective lipid profile often ascribed to *APOC3* mutation carriership is most pronounced in c.55+1G>A and p.Ala43Thr. C.-13-2A>G mutation carriers show a trend towards lower triglyceride levels and HDL-C percentiles. ApoCIII levels are significantly lower in c.-13-2A>G mutation carriers, but not in c.55+1G>A or p.Ala43Thr mutation carriers. ApoCIII isoform distribution did not differ between carriers and controls.

ApoCIII resides on both HDL and apoB-containing particles and acts on triglyceride metabolism by inhibiting lipoprotein lipase (LPL) mediated lipolysis. ApoCIII inhibits hepatic uptake of apoB-containing lipoproteins, enhances catabolism of HDL particles, enhances monocyte adhesion to vascular endothelial cells and activates inflammatory signalling pathways. Our findings are in line with the notion that heterozygosity for *APOC3* mutations is associated with a favourable lipid profile. Not all of the lipid parameters reached significance, probably due to the low number of study participants in each group.

Previously, Liu and co-workers have reported that the mutation p.Ala43Thr (p.Ala-23Thr) leads to less efficient lipid binding, resulting in degradation of free apoCIII. They report that the mutation is phenotypically associated with low plasma triglyceride levels in two of three mutation carriers. This is in agreement with our findings. Interestingly, however, apoCIII levels were substantially lower than in our p.Ala43Thr mutation carriers (1.75 vs 4.92 mg/dl). Triglyceride levels were in the same range (0.74 vs 0.58 mmol/l), whereas HDL-C levels were higher in our carriers (1.14 vs 2.19 mmol/l). These differences cannot be attributed to age, gender or BMI.

Sundaram and co-workers set out to investigate the effect of the p.Ala43Thr mutation on lipid metabolism and report that in McA-RH7777 cells transfected with the mutant apoCIII protein, fusion of lipid droplets with apoB in the VLDL assembly pathway is impaired. It was therefore postulated that the absence of a structural element within the N-terminal region of the apoCIII protein is responsible for the loss of function of apoCIII due to the p.Ala43Thr mutation.

The c.55+1G>A and c.-13-2G>A are novel variants located respectively in intron 2 and 1 of *APOC3* and affecting splice sites. Both mutations are predicted to disrupt a 5’ splicing site. To the best of our knowledge, the consequences of these mutations have not been reported. Plasma apoCIII levels decreased to a similar extent in c.55+1G>A and c.-13G>A mutation carriers as compared to p.Ala43Thr mutation carriers. Plasma HDL-C levels and triglycerides of c.55+1G>A mutation carriers are in the range of p.Ala43Thr carriers and show a strong atheroprotective phenotype. The atheroprotective phenotype is less pronounced...
in c.-13-2G>A mutation carriers. However, it is noteworthy that HDL-C levels in both c.-13-2G>A carriers and family controls are above the 85\textsuperscript{th} percentile. A possible explanation pertains to the fact that the control group consists of females only, naturally displaying higher HDL-C levels, whereas the carrier group consists of two males and two females.

ApoCIII has three different isoforms characterised by zero, one or two sialic acid residues. ApoCIII\textsubscript{1} and apoC-III\textsubscript{2} inhibit lipoprotein lipase (LPL), and are therefore crucial for triglyceride clearance. However, apoC-III\textsubscript{0} has limited capacity to inhibit LPL and thus increases triglyceride clearance capacity.\textsuperscript{34,35} Mutations in \textit{N-acetylgalactosaminytransferase 2} (\textit{GALNT2}) have been shown to cause a reduction of ppGalNAc-T2, leading to posttranslational modification of apoCIII, increased plasma levels of apoCIII\textsubscript{0}, decreased plasma levels of apoCIII\textsubscript{1} and apoCIII\textsubscript{2} and improved postprandial triglyceride clearance.\textsuperscript{35} These data have established that apoCIII isoforms are key regulators of triglyceride metabolism and potentially key players in atherogenesis. In our cohort, apoCIII isoform distribution does not differ between cases and controls, which indicates that post translational modification is not influenced by the mutations described in this study.

The differences in apoCIII between carriers and non carriers were not associated with differences in apoCIII distribution among HDL and TG-rich apoB-containing lipoproteins. In contrast, von Eckardstein and co workers report that in a family with a Lys58Glu mutation, carriership was associated with a larger decrease of apoCIII in VLDL (-85\%) compared to HDL (-75\%).\textsuperscript{36} Furthermore, we did not find a change in HDL particle size in our \textit{APOC3} mutation carriers in contrast to the finding of larger HDL particles in Lys58Glu carriers by von Eckardstein and co workers. These discrepancies may be due to inclusion of different mutations, affecting different regions of the \textit{APOC3} gene.

Elevated plasma levels of low density lipoprotein cholesterol (LDL-C) and triglycerides are important contributors to premature coronary heart disease.\textsuperscript{37–39} Whereas the role of LDL-C in cardiovascular disease (CVD) is established, the role of triglycerides is more controversial. This is partly due to its inextricable linkage to HDL-C, a biomarker inversely correlated to CVD. Furthermore, plasma triglycerides are associated with traditional cardiovascular risk factors such as obesity and diabetes, thereby constituting a source of potential confounding. However, the Adult Treatment Panel (ATP) III guidelines has defined the normal range of triglycerides as <150 mg/dL (1.69 mmol/L).\textsuperscript{40} Furthermore, the ATPIII recommends that non-HDL cholesterol (LDL+VLDL, be used as a secondary target of therapy, after LDL-C lowering. Genetic studies are widely considered a powerful tool to study the influence of a biomarker on endpoints, since confounding factors are circumvented and lifelong exposure to the biomarker is guaranteed. We used this approach and show that carriers of \textit{APOC3} mutations are characterized by high levels of plasma HDL-C and low levels of plasma triglycerides. The majority of evidence indicates that this apparently favourable lipid profile translates into a low CVD risk. There is ample evidence that apoCIII might be a suita-
Identification of two novel APOC3 mutations

Plasma apoCIII levels have been shown to be associated with coronary artery disease, myocardial infarction, coronary progression in niacin treated subjects, presence of lesions in lovastatin treated patients and cardiovascular disease in type II diabetic patients. In only one study, plasma apoCIII was inversely associated with CAD. The lack of power due the small size of our patient cohort precludes us from observing any effect of APOC3 mutation carrierrship on cardiovascular endpoints.

By confining our sequence efforts to participants with plasma HDL levels >95th percentile, we introduced a selection bias and may have missed APOC3 mutations not resulting in the expected phenotype. Furthermore, we present data on a small cohort of study participants. This is however inherent to family studies and the results observed are consistent within each group. Recruitment of family controls enhanced the power of the study. Last, given the linkage disequilibrium of APOC3 with APOA1 and APOA5, we cannot exclude the possibility that the lipid changes ascribed to APOC3 mutation carrierrship, are in fact attributable to APOA4 or APOA5, which are known to influence HDL and triglyceride metabolism. However, in this study we describe three rare variants leading to a loss of function of APOC3, indicating that changes in lipid profiles can be ascribed to altered APOC3 function. This is supported by the fact that interventions aimed at decreasing APOC3, result in an HDL-C increase and triglyceride decrease.

In summary, we present data on two novel mutations in APOC3, as well as one previously identified mutation. Two of these three mutations show a clear effect on lipid profiles, accompanied by the relatively large decreases in plasma apoCIII, whereas the third mutation is associated with less pronounced changes in the lipid profile and smaller decrease in plasma apoCIII. This study suggests an inverse association between apoCIII and favourable lipid profiles and lends support to the study the effects of apoCIII lowering therapy.

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Chapter 4


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