Apolipoprotein A-V, triglycerides and risk of coronary artery disease: the prospective Epic-Norfolk Population Study

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Abstract In mouse models, apolipoprotein A-V (apoA-V) exhibits triglyceride (TG)-lowering effects. We investigated the apoA-V/TG relationship and the association of apoA-V with coronary artery disease (CAD) risk by determining serum apoA-V levels and genotypes in a nested case-control (n = 1,034/2,031) study. Both univariate and multivariate apoA-V levels showed no association with future CAD (P = 0.4 and 0.5, respectively). Unexpectedly, there was a significant positive correlation between serum apoA-V and TG in men and women (r = 0.36 and 0.28, respectively, $P <$ 0.001 each) but a negative correlation between apoA-V and LPL mass (r = −0.14 and −0.12 for men and women respectively, $P <$ 0.001 each). The frequency of the c.56C>G polymorphism did not differ between cases and controls despite significant positive association of c.56G with both apoA-V and TG levels. For −1131T>C, the minor allele was significantly associated with lower apoA-V yet higher TG levels and was overrepresented in cases (P = 0.047). The association of −1131T>C with CAD risk, however, was independent of apoA-V levels and likely acts through linkage disequilibrium with *APOC3* variants. The positive correlation of apoA-V levels with TG levels, negative correlation with LPL levels, and lack of association with CAD risk highlight the need for further human studies to clarify the role of apoA-V.—Vaessen, S. F. C., F. G. Schaap, J-A. Kuivenhoven, A. K. Groen, B. A. Hutten, S. M. Boekholdt, H. Hattori, M. S. Sandhu, S. A. Bingham, R. Luben, J. A. Palmen, N. J. Wareham, S. E. Humphries, J. J. P. Kastelein, P. J. Talmud, and K-T. Khaw. **Apolipoprotein A-V, triglycerides and risk of coronary artery disease: the prospective Epic-Norfolk Population Study. *J. Lipid Res.* 2006.** 47: **2064–2070.**

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Epidemiological studies show that increased serum triglyceride (TG) levels are associated with an increased risk of coronary artery disease (CAD) (1, 2). The *APOA1/C3/A4/A5* gene cluster on chromosome 11 has been shown to have a strong effect on plasma TG levels (3, 4). The newest member of this cluster, *APOA5*, was discovered in 2001 by two groups (5, 6). The gene is expressed in the liver and encodes an apolipoprotein A-V (apoA-V) that in the circulation is associated with VLDL, HDL, and chylomicrons (6, 7). Studies in genetically engineered mice revealed a prominent role for apoA-V in TG metabolism: overexpression of the human or murine gene gave rise to a marked decrease of serum TG levels (5, 8, 9), whereas mice lacking *APOA5* displayed a 4-fold increase in serum TG levels (5, 10). The exact mechanism by which apoA-V affects TG levels is unclear, but it is currently thought to augment both VLDL-TG hydrolysis and hepatic uptake of lipoprotein core remnants (10–14).

Suggestions that apoA-V plays a significant role in humans are predominantly based on genetic association studies. Three common haplotypes have been identified: wild-type haplotype *APOA5*1*, *APOA5*2* (defined by rare alleles of −1131T>C, c.−3A>G, IVS3+476G>T, and c.1259T>C), and *APOA5*3* (defined by a rare allele of c.56C>G) (15). Carriers of the two single nucleotide polymorphisms (SNPs) representing these haplotypes, −1131T>C for *APOA5*2* and c.56C>G (S19W) for *APOA5*3*, have consistently been

Abbreviations: apoA-V, apolipoprotein A-V; BMI, body mass index; CAD, coronary artery disease; CI, confidence interval; EPIC, European Prospective Investigation into Cancer and Nutrition; OR, odds ratio; SNP, single nucleotide polymorphism; TG, triglyceride.

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described to present with higher plasma TG levels and an atherogenic lipid profile (e.g., increased levels of small, dense LDLs) (15–18). Further support that apoA-V plays an important role in human TG metabolism comes from individuals with point mutations in the *APOA5* gene, resulting in premature truncation of the apoA-V protein, who develop severe hypertriglyceridemia and/or hyperchylomicronemia (19–21).

Because *APOA5* gene variation is associated with effects on TG levels, apoA-V can be hypothesized to influence the risk of CAD. This hypothesis has been tested in a large number of genetic association studies, and the results are equivocal: whereas several studies claim polymorphisms in the *APOA5* gene, especially −1131T>C, to be associated with increased susceptibility to or progression of CAD (17, 22–27), others report no association with CAD (28–30). These studies, however, suffered from a lack of data on the effects of genetic variation on apoA-V plasma levels. Hence, having the availability of both serum apoA-V measurements and genotype information on the −1131T>C and c.56C>G SNPs, we set out to explore the relationships between apoA-V, lipid parameters, and the risk of future CAD in a case-control study nested in the European Prospective Investigation into Cancer and Nutrition (EPIC) Norfolk Population Study.

METHODS

Study design

We performed a nested case-control study among participants in the EPIC-Norfolk cohort study, a population of 25,663 men and women between 45 and 79 years of age (31). EPIC-Norfolk is part of the 10 country collaborative EPIC Study designed to investigate determinants of cancer. From the outset, additional data were obtained to enable the assessment of determinants of other diseases. Recruitment of participants was done by mail from age-sex registers of general practices. At the baseline survey between 1993 and 1997, participants completed a detailed health and lifestyle questionnaire and attended a clinic visit at which additional data, including anthropometry, blood pressure, and a nonfasting blood sample, were collected by trained nurses using standardized protocols as described previously (31). All individuals have been flagged for mortality at the UK Office of National Statistics, with vital status ascertained for the entire cohort. Death certificates for all decedents were coded by trained nosologists according to the International Classification of Diseases (ICD) ninth revision. Death was considered attributable to CAD if the underlying cause was coded as ICD 410–414. In addition, participants admitted to the hospital were identified using their unique National Health Service number by data linkage with ENCORE (the East Norfolk Health Authority database), which identifies all hospital contacts throughout England and Wales for Norfolk residents. The study was approved by the Norwich District Health Authority Ethics Committee, and all participants gave signed informed consent.

All participants who reported a history of heart attack or stroke at the baseline clinic visit were excluded. Cases were individuals who developed a fatal or nonfatal CAD during follow-up until November 2003. Controls were study participants who remained free of any cardiovascular disease during follow-up. Two controls were matched to each case by sex, age (within 5 years), and time of enrollment (within 3 months).

Biochemical and DNA analyses

Serum levels of total cholesterol, HDL-cholesterol, and TG were measured with the RA 100 (Bayer Diagnostics, Basingstoke, UK), and LDL-cholesterol levels were calculated using the Friedewald formula. Measurement of LPL concentration was performed with a commercially available ELISA (Dainippon). Serum apoA-V levels were measured using a newly developed sandwich ELISA using monoclonal antibodies as described previously (32). Briefly, plates were coated overnight with monoclonal antibody B10E (100 μl of 1.2 μg/ml in 0.05 M carbonate buffer, pH 9.6) at 4°C. After washing with PBX (PBS containing 0.1% Triton X-100), wells were blocked for 2 h at room temperature with PBXC (PBX containing 1% casein; Hammerstan grade; Merck). After washing, wells were incubated for 2 h with 100 μl standards, reference sera, and samples (1:100) all diluted in PBXC. Next, wells were washed and incubated with 100 μl of biotinylated monoclonal antibody E8E (1.0 μg/ml in PBXC) for 1 h. After washing, wells were incubated with streptavidin-horseradish peroxidase (Dako; 1:3,000 in PBXC) for 1 h. After extensive washing, the plate was then incubated with 3,3′,5,5′-Tetramethylbenzidine substrate (Pierce). The color reaction was stopped after exactly 30 min with 2 M sulfuric acid, and absorbance was read at 450 nm (Easia reader; Medgenix Diagnostics). On every plate, the same three reference sera were included, resulting in an interassay variation of 7.0 ± 0.9%.

*APOA5 g*enotyping (c.56C>G and −1131T>C) was performed using Taqman allelic discrimination with specific VIC-labeled probes for the wild-type alleles and FAM-labeled probes for the minor alleles. For c.56C>G, forward primer 5′-ccaggccctgattacctagtc-3′, reverse primer 5′-gaagtagtcccagaagcctttcc-3′, and probes FAM-5′-agcgttttgggccac-3′ and VIC-5′-cagcgttttcggccac-3′ were used; for −1131T>C, we used forward primer 5′-ccctgcgagtggagttca-3′, reverse primer 5′-ctctgagccccaggaactg-3′ and probes FAM-5′-agcgaaagtaagattt-3′ and VIC-5′-agcgaaagtgagattt-3′.

For all assays, samples were analyzed in random order to avoid systemic bias. Researchers and laboratory personnel were blinded to identifiable information and could identify samples by number only.

Statistical analysis

Baseline characteristics were compared between cases and controls using a mixed-effect model for continuous variables or conditional logistic regression for categorical variables, both taking into account matching for sex, age, and enrollment time. Because serum TG, LPL, and apoA-V levels had a skewed distribution, values were log-transformed before being used in statistical analyses as continuous variables. Serum apoA-V levels were categorized in quartiles based on men and women separately. Linear associations between apoA-V quartiles and traditional risk factors were calculated using linear regression for continuous variables and Chi-square tests for categorical variables. Pearson's correlation coefficients and corresponding *P* values were calculated to assess associations between log-transformed serum apoA-V levels and established continuous CAD risk factors. The association of serum apoA-V levels with future CAD was determined using a conditional logistic regression model, both univariate and multivariate, with adjustment for the following cardiovascular risk factors: diabetes (yes or no), smoking (never, past, current), body mass index (BMI), systolic blood pressure, diastolic blood pressure, total cholesterol, and HDL-cholesterol. In addition, odds ratio (OR) and corresponding 95% confidence interval (CI) as an estimate of the relative risk of

TABLE 1. Baseline characteristics of future coronary artery disease cases and matched controls in the European Prospective Investigation into Cancer and Nutrition-Norfolk population cohort

Characteristic	Controls (n = 2,031)	Cases (n = 1,034)	*P*
Male sex	63 (1,271)	63 (654)	Matched
Age (years)	65.3 ± 7.8	65.5 ± 7.8	Matched
Diabetes	1.8 (36)	6.8 (70)	<0.0001
Smoking			
Current	8 (169)	15 (156)	<0.0001
Previous	51 (1,025)	52 (531)	
Never	41 (816)	33 (335)	
BMI (kg/m^2)	26.3 ± 3.5	27.3 ± 3.9	<0.0001
Diastolic blood pressure (mmHg)	83.5 ± 11.0	85.9 ± 11.9	<0.0001
Systolic blood pressure (mmHg)	139.0 ± 17.8	144.0 ± 18.8	<0.0001
Total cholesterol (mmol/l)	6.3 ± 1.2	6.5 ± 1.2	<0.0001
LDL-cholesterol (mmol/l)	4.1 ± 1.0	4.3 ± 1.1	<0.0001
HDL-cholesterol (mmol/l)	1.4 ± 0.4	1.3 ± 0.4	<0.0001
TG (mmol/l)	1.7 (1.2–2.3)	1.9 (1.4–2.8)	<0.0001
Lipoprotein lipase (ng/ml)	66 (46–92)	61 (43–85)	<0.0001
ApoA-V (ng/ml)	181 (144–236)	185 (141–242)	0.3

apoA-V, apolipoprotein A-V; BMI, body mass index; TG, triglyceride. Data are presented as means ± SD, percentage or median (interquartile range). Significance was calculated using conditional logistic regression or mixed-effect modeling considering matching for age, gender, and enrollment time, where appropriate.

incident CAD were calculated using conditional logistic regression analysis, which takes into account the matching for sex, age, and enrollment time. The lowest apoA-V quartile was used as the reference category. Differences in the distribution of SNP carriers (heterozygous and homozygous pooled) were calculated using Chi-square analysis. Furthermore, to estimate the relative risk of incident CAD associated with carriage of the minor allele, we calculated OR and corresponding 95% CI using conditional logistic regression. Additionally OR and 95% CI were calculated with additional adjustment for serum apoA-V levels and for both serum apoA-V and TG levels. Differences in apoA-V serum levels between SNP carriers and noncarriers were determined using ANOVA. Statistical analyses were performed using SPSS software (version 12.0.1; Chicago, IL). A two-tailed $P < 0.05$ was considered statistically significant.

RESULTS

Baseline characteristics

Cases were 1,034 participants who were apparently healthy at baseline but developed fatal or nonfatal CAD during an average 6 year follow-up. Of these cases, 997 could be matched with 2 controls and the remainder with 1 control, making a total of 2,031 controls. Matching was by age, sex, and enrollment time. As expected, conventional cardiovascular risk factors were increased significantly in cases compared with controls (**Table 1**). Serum apoA-V levels, however, were not significantly different between cases and controls: 181 (144–236) versus 185 (141–242) ng/ml, respectively ($P = 0.3$). Of note, women presented with significantly higher serum apoA-V levels compared with men: 195 (154–254) versus 175 (138–226) ng/ml, respectively ($P < 0.001$).

ApoA-V serum levels and cardiovascular risk factors

Given the difference in apoA-V levels between men and women, associations between serum apoA-V levels and cardiovascular risk factors were examined for men and women separately (**Table 2**). In both sexes, apoA-V levels were positively correlated with BMI, diabetes, total cholesterol, HDL-cholesterol, and TG. However, apoA-V levels were negatively correlated with serum LPL concentration ($r = -0.14$ and -0.12 for men and women, respectively,

TABLE 2. Distribution of cardiovascular risk factors by serum apoA-V quartiles

Risk Factor	ApoA-V Quartile 1	2	3	4	P^a	r^b	P^b
Men (case/control)	172/337	168/348	169/358	187/321			
ApoA-V range (ng/ml)	<138	138–175	176–227	>227			
BMI (kg/m^2)	26.1 ± 2.9	26.4 ± 3.3	26.8 ± 3.2	27.4 ± 3.5	<0.0001	0.156	<0.0001
Diabetes	3.5 (18)	3.1 (16)	3.4 (18)	6.5 (33)	<0.05		
Total cholesterol (mmol/l)	5.9 ± 1.1	6.0 ± 1.1	6.3 ± 1.1	6.4 ± 1.1	<0.0001	0.179	<0.0001
LDL-cholesterol (mmol/l)	4.0 ± 1.0	4.0 ± 1.0	4.1 ± 1.0	4.0 ± 0.9	0.915	0.006	0.789
HDL-cholesterol (mmol/l)	1.2 ± 0.3	1.3 ± 0.3	1.3 ± 0.4	1.2 ± 0.3	<0.001	0.078	<0.001
TG (mmol/l)	1.6 ± 0.7	1.7 ± 0.8	2.1 ± 1.0	2.8 ± 1.5	<0.0001	0.379	<0.0001
Lipoprotein lipase (ng/ml)	74 ± 55	68 ± 40	65 ± 45	59 ± 37	<0.0001	−0.140	<0.0001
Women (case/control)	104/192	99/208	95/206	102/200			
ApoA-V range (ng/ml)	<153	153–194	195–254	>254			
BMI (kg/m^2)	26.0 ± 4.1	26.3 ± 4.1	26.5 ± 4.1	27.4 ± 4.7	<0.001	0.107	<0.0001
Diabetes	1.4 (4)	1.0 (3)	2.0 (6)	5.3 (16)	<0.01		
Total cholesterol (mmol/l)	6.4 ± 1.2	6.7 ± 1.1	6.9 ± 1.2	6.9 ± 1.3	<0.0001	0.202	<0.0001
LDL-cholesterol (mmol/l)	4.2 ± 1.1	4.4 ± 1.0	4.5 ± 1.1	4.3 ± 1.1	0.142	0.056	0.058
HDL-cholesterol (mmol/l)	1.4 ± 0.4	1.5 ± 0.4	1.6 ± 0.4	1.6 ± 0.5	<0.0001	0.139	<0.0001
TG (mmol/l)	1.6 ± 0.8	1.7 ± 0.7	1.9 ± 1.0	2.3 ± 2.0	<0.0001	0.255	<0.0001
Lipoprotein lipase (ng/ml)	95 ± 52	95 ± 55	89 ± 54	80 ± 52	<0.05	−0.124	<0.001

Data are presented as means ± SD or percentage (n) per apoA-V quartile.
[a] Significance for linearity between serum apoA-V quartiles and risk factor levels.
[b] Pearson's correlation between log-transformed serum apoA-V levels and risk factor levels with the corresponding *P* value.

TABLE 3. ORs for future coronary artery disease according to serum apoA-V quartile

Parameter	ApoA-V Quartile 1	2	3	4	*P* for Linearity
Men (cases/controls)	172/337	168/348	169/358	187/321	
Range (ng/ml)	<138	138–175	176–227	>227	
Model 1	1.00	0.99 (0.75–1.30)	0.95 (0.72–1.25)	1.14 (0.87–1.49)	0.3
Model 2	1.00	1.00 (0.74–1.35)	0.88 (0.64–1.20)	1.00 (0.73–1.37)	0.7
Women (cases/controls)	104/192	99/208	95/206	102/200	
Range (ng/ml)	<153	153–194	195–254	>254	
Model 1	1.00	0.94 (0.66–1.32)	0.90 (0.63–1.27)	0.99 (0.70–1.39)	0.9
Model 2	1.00	0.95 (0.65–1.41)	0.83 (0.56–1.24)	0.86 (0.57–1.30)	0.3

CI, confidence interval; OR, odds ratio. Data are ORs and corresponding 95% CIs. Model 1 was calculated by conditional logistic regression, taking into account matching for age, sex, and enrollment time. Model 2 was as model 1 and additionally adjusting for diabetes, smoking, BMI, diastolic and systolic blood pressure, total cholesterol, and HDL-cholesterol.

$P < 0.001$ for each). The positive correlation between serum apoA-V levels and serum TG levels was stronger than that for any other parameter ($r = 0.36$ and 0.28 for men and women, respectively, $P < 0.001$ for each). Notably, no correlation was observed between apoA-V and LDL-cholesterol levels.

ApoA-V serum levels, *APOA5* genotypes, and risk of future CAD

The relationship between apoA-V levels and future CAD was first evaluated in a univariate model using conditional logistic regression, taking into account matching for age, sex, and enrollment time. When apoA-V serum levels were entered as the continuous variable, no association was observed with risk of future CAD (OR = 1.08 per 1 ng/ml, 95% CI = 0.90–1.31, $P = 0.4$). Second, multiple stepwise backward regression analysis identified diabetes, smoking, BMI, diastolic and systolic blood pressure, total cholesterol, and HDL-cholesterol as confounding variables. Using multivariate analysis with adjustment for these variables, apoA-V levels remained not associated with CAD (OR = 0.93 per 1 ng/ml, 95% CI = 0.74–1.16, $P = 0.5$). Consistently, OR for future CAD did not differ from unity across apoA-V quartiles for both men and women, either unadjusted or adjusted for the confounding variables mentioned above (**Table 3**).

To extend this analysis, all study participants were genotyped for −1131T>C and c.56C>G (S19W), representing the haplotypes *APOA5*2* and *APOA5*3*, respectively. Because of the low numbers of homozygotes for the minor alleles (8 for c.56G and 13 for −1131T>C), heterozygotes and homozygotes for the minor alleles (minor allele carriers) were pooled, unless stated otherwise. For the functional c.56C>G polymorphism, the frequency of minor allele carriers was not significantly different between cases and controls (11.4% and 11.5%, respectively; $P = 1.0$) (**Table 4**), with no difference of effect in men and women (data not shown). Minor allele (c.56G) carriers presented with increased serum apoA-V levels compared with homozygotes for the wild-type allele (279 ± 150 ng/ml and 192 ± 85 ng/ml, respectively; $P < 0.001$). Notably, heterozygote and homozygote carriers of the minor c.56G allele had 43% and 105% higher apoA-V levels, respectively, compared with homozygotes for the wild-type allele ($P < 0.001$ overall). In agreement with the positive correlation between apoA-V and TG levels in the entire cohort, minor allele carriers presented with significantly higher serum TG levels compared with homozygotes for the wild-type allele (2.3 ± 1.9 and 2.0 ± 1.1 mmol/l, respectively; $P = 0.001$). In contrast, there was no association between the c.56C>G genotype and serum LPL concentration.

TABLE 4. Distribution of *APOA5* genotypes for c.56C>G and −1131T>C in cases and controls

c.56C>G	CC	CG and GG	Minor Allele Frequency	*P*[a]	OR (95% CI)[b] Model 1	Model 2	Model 3
Controls (n)	1,543 (88.5%)	200 (11.5%)	0.059	1.0	0.97 (0.74–1.25)	0.95 (0.72–1.24)	1.01 (0.77–1.33)
Cases (n)	800 (88.6%)	103 (11.4%)	0.059		$P = 0.8$	$P = 0.7$	$P = 1.0$
−1131T>C	**TT**	**TC and CC**	**Minor Allele Frequency**	***P*[a]**	**OR (95% CI)[b] Model 1**	**Model 2**	**Model 3**
Controls (n)	1,559 (89.3%)	186 (10.7%)	0.056	0.047	1.29 (1.00–1.67)	1.30 (1.01–1.69)	1.12 (0.86–1.47)
Cases (n)	783 (86.7%)	115 (13.3%)	0.069		$P = 0.055$	$P = 0.046$	$P = 0.4$

Data are presented as cases (n) and percentage within subgroup. Model 1 was calculated by conditional logistic regression, taking into account matching for age, sex, and enrollment time. Model 2 was as model 1, including adjustment for serum apoA-V levels. Model 3 was as model 2, additionally adjusted for serum TG levels.

[a] Differences in genotype frequency (heterozygous and homozygous carriers for the minor allele combined) calculated using Chi-square analysis.

[b] OR and 95% CI calculated for minor allele carriers (heterozygotes and homozygotes combined), using common allele homozygotes as the reference category.

For the −1131T>C polymorphism, carriers of the minor allele were overrepresented in future CAD cases compared with controls (13.3% and 10.7%, respectively), which reached statistical significance ($P < 0.05$) (Table 4), and with similar magnitude for both men and women (data not shown). Compared with homozygotes for the wild-type allele, minor allele carriers presented with increased serum TG levels (2.4 ± 1.4 and 2.0 ± 1.2 mmol/l, respectively; $P < 0.001$), yet with decreased apoA-V levels (183 ± 120 vs. 205 ± 95 ng/ml; $P < 0.001$). Importantly, however, the association between the −1131T>C allele and increased risk of CAD was unchanged after adjusting for apoA-V levels (Table 4). However, when additionally adjusting for TG levels, the −1131T>C minor allele was no longer significantly associated with an increased risk of future CAD. Like the c.56C>G polymorphism, the −1131T>C SNP was not associated with serum LPL levels.

DISCUSSION

Contrary to expectations based on studies in individuals bearing nonsense mutations in the *APOA5* gene and studies in genetically engineered mice, the results from this large prospective case-control study show that serum apoA-V levels are positively correlated with serum TG levels yet are not associated with the risk of future CAD.

ApoA-V serum level and TGs

The positive correlation between apoA-V and TG levels observed in the current analysis agrees with a recent study by Dallinga-Thie et al. (33) in patients with type 2 diabetes yet contrasts with two initial studies showing a weak negative correlation between these parameters (7, 32). Differences in lifestyle and genetic background, but especially study size (n = 196 and 40, respectively, compared with 3,065 in this analysis) may underlie the discrepancy between these two studies (7, 32) and our results. The current result was unexpected, given the reported hypotriglyceridemic effects of apoA-V in animal models (5, 8, 10, 12). Although care should be taken when using epidemiological data to comment on complex (lipolytic) pathways, our data do not support the idea that serum apoA-V levels are a good indicator of plasma TG hydrolysis, as recently proposed (13, 14). We hypothesize that the interplay between many key parameters, including apoC-II, apoC-III, lipoprotein lipase, and the numerous lifestyle factors known to affect TG metabolism, prevent apoA-V from standing out in the current analysis. One example in support of this hypothesis is the finding that adjustment for apoC-III, a negative regulator of LPL hydrolysis, attenuates the positive association of apoA-V levels with TG levels in hypertriglyceridemic patients (F. G. Schaap, unpublished results). This hypothesis is further supported by studies showing that the clinical manifestation of severe dyslipidemia among carriers of an *APOA5*-truncating mutation is entirely dependent on additional effects mediated by the second common TG-increasing allele or on other factors such as age, glucose levels, and obesity (19–21). Furthermore, the remaining masked etiology of severe hypertriglyceridemia after exclusion of known TG-modulating factors in the majority of patients diagnosed with this disorder suggests that additional modulators of TG metabolism remain unidentified. Thus, despite clear evidence from animal and in vitro studies that apoA-V has a major TG-lowering effect, this study shows that serum apoA-V levels are in fact positively correlated with serum TG in the general population.

ApoA-V and risk for CAD

Despite the positive association of serum apoA-V levels with TG levels, multivariate analysis adjusting for all identified confounding factors clearly revealed the absence of an association between serum apoA-V levels and the risk of future CAD. In this analysis, we could not account for some main regulators of plasma TG hydrolysis, such as apoC-II and apoC-III concentrations; however, we did have access to LPL serum concentration, a parameter that was recently shown to be associated with lower TG levels and a reduced risk of CAD (34). Interestingly, in this analysis, apoA-V concentration and serum LPL concentration appeared to be inversely correlated, for which we have no explanation. It can be hypothesized that apoA-V stabilizes the binding of the catalytically active dimeric form of LPL to the vascular endothelium (13), thereby preventing the release of LPL monomers into the circulation. However, this is in complete disagreement with the idea that increased serum LPL concentration somehow reflects whole-body LPL production or the systemic potential to hydrolyze plasma TG (34). In fact, the individuals with the highest TG and apoA-V levels present with the lowest serum LPL concentration. This example shows that it is very difficult to explain how plasma TG hydrolysis occurs on the basis of plasma levels of key proteins, enzymes, and lipids. Intricate biochemical experiments may give better clues, but these experiments in turn are hampered by the fact that TG hydrolysis occurs at the endothelium adipose tissue and the cardiac and skeletal muscle, tissues in which LPL is known to be differentially regulated (35).

In contrast to our data on apoA-V serum levels, several genetic association studies suggest the presence of a relationship between *APOA5* and the risk of future CAD (17, 22, 24, 25, 27). To allow for a direct comparison of the EPIC cohort with these studies, we genotyped the cohort for −1131T>C and c.56C>G. In addition, this also enabled investigation of the relationships between *APOA5* genotype, serum apoA-V levels, and risk of future CAD. The allele frequencies of both c.56C>G and −1131T>C were similar to those reported previously (15, 17, 36). Carrying the minor allele of the signal peptide variant c.56C>G was associated with both increased apoA-V and increased TG levels, confirming recent findings by Dallinga-Thie et al. (33) in patients with type 2 diabetes. Intriguingly, this functional signal peptide SNP has been reported to reduce the secretion potential of the resulting c.56G variant in vitro (37). Assuming that this is also true in vivo, this variant is either less efficiently bound to the

heparan sulfate proteoglycans on the vessel wall, resulting in higher serum levels, or may be catabolized less efficiently than the wild-type variant. Confirming the main finding in the total cohort, carriers of the c.56G allele, although presenting with increased apoA-V and TG levels, showed unchanged risk of future CAD.

The idea that apoA-V is associated with CAD is based primarily on association studies focusing on the −1131T>C variant (17, 22, 24, 25). In agreement, the current study also shows that the −1131T>C variant was slightly, yet significantly, overrepresented in future CAD cases. Importantly, however, we show here that the effect of this SNP on CAD risk was independent of serum apoA-V levels, because adjustment for apoA-V levels did not attenuate the increased risk of future CAD. In this context, it is of note that the −1131T>C promoter polymorphism has been reported not to influence *APOA5* gene transcription directly (37–39). Moreover, the three other *APOA5* SNPs, which together with −1131T>C define haplotype *APOA5*2*, also appear to be nonfunctional (35). Finally, this *APOA5* polymorphism is in strong linkage disequilibrium with the *APOC3* variant −482C>T, which has been reported to disrupt the insulin-responsiveness of the *APOC3* gene (40). In the absence of an inverse relation between apoA-V and TG levels, we observed that the association between −1131T>C and the risk of CAD was mediated by TG levels. Together, our data suggest that the association of −1131T>C with future CAD is likely attributable to linkage disequilibrium with *APOC3* variants or to other closely linked genetic variations. These findings also illustrate the complexity of analyzing the contribution of the *APOA1/C3/A4/A5* gene cluster to lipid metabolism and CAD risk.

Limitations of the study

Several aspects of the current study warrant attention. First, CAD events were ascertained through death certification and hospital admission data, which may lead to both underascertainment and misclassification of cases. Previous validation in this cohort, however, indicates high specificity of such case ascertainment (41). Second, levels of apoA-V and other lipid-related variables were determined in nonfasting serum that was obtained at a nonuniform time of the day (related to the fact that >26,000 individuals were enrolled). Diurnal variation, variation over time, and differences in the time span since the last meal are likely to have affected these variables. The latter is especially true for TG levels. This fact makes comparisons with other studies of apoA-V levels in fasting plasma difficult. However, in the Western world, people live under constant "postprandrial" conditions, and studies into the associations between lipids, lipoproteins, and CAD risk may be more physiologically relevant under nonfasting conditions. Furthermore, random measurement error in both case ascertainment and time variation would lead to an underestimation of any relationships between risk factors and CAD risk. The extent of measurement error in our study, however, is unlikely to differ from that for other risk factors or from other prospective studies.

Conclusions

The data of this prospective analysis show that serum apoA-V levels are positively related to TG levels and negatively associated with LPL levels, yet not with increased risk of future CAD. These results are supported by analysis of the functional c.56C>G polymorphism. This study furthermore provides evidence that the association of the −1131T>C promoter polymorphism with increased CAD risk is independent of serum apoA-V levels. Together, the results from this study highlight the need for additional human studies to clarify the functional role of apoA-V.

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