Evidence for a complex relationship between apoA-V and apoC-III in patients with severe hypertriglyceridemia

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Abstract The relevance of apolipoprotein AV (apoA-V) for human lipid homeostasis is underscored by genetic association studies and the identification of truncation-causing mutations in the APOA5 gene as a cause of type V hyperlipidemia, compatible with an LPL-activating role of apoA-V. An inverse correlation between plasma apoA-V and triglyceride (TG) levels has been surmised from animal data. Recent studies in human subjects using (semi)quantitative immunoassays, however, do not provide unambiguous support for such a relationship. Here, we used a novel, validated ELISA to measure plasma apoA-V levels in patients (n = 28) with hypertriglyceridemia (HTG; 1.8–78.7 mmol TG/l) and normolipidemic controls (n = 42). Unexpectedly, plasma apoA-V levels were markedly increased in the HTG subjects compared with controls (1,987 vs. 258 ng/ml; P < 0.001). In the HTG group, apoA-V and TG were positively correlated (r = +0.44, P = 0.02). In addition, we noted an increased level of the LPL-inhibitory protein apoC-III in the HTG group (45.8 vs. 10.6 mg/dl in controls; P < 0.001). The correlation between apoA-V and TG levels in the HTG group disappeared (partial r = +0.09, P = 0.65) when controlling for apoC-III levels. In contrast, apoC-III and TG remained positively correlated in this group when controlling for apoA-V (partial r = +0.43, P = 0.025). Our findings suggest that in HTG patients, increased TG levels are accompanied by high plasma levels of apoA-V and apoC-III, apolipoproteins with opposite modes of action. This study provides evidence for a complex interaction between apoA-V and apoC-III in patients with severe HTG.

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The recognition of hypertriglyceridemia (HTG) as an independent risk factor for cardiovascular pathologies (1) necessitates the identification of the factors involved in the regulation of plasma triglyceride (TG) levels. Along with esterified cholesterol, TGs constitute the neutral lipid core of chylomicrons, VLDL, and their remnants. LPL is the principal enzyme involved in the degradation of TG in plasma. The hydrolytic action of LPL requires the presence of a cofactor [i.e., apolipoprotein C-II (apoC-II)] and is modulated by a number of other factors (2–4). Important negative regulators are apoC-III and the recently identified angiopterin-like proteins ANGPTL3 and ANGPTL4 (3, 5–7). In addition to these negative effectors, the novel apolipoprotein apoA-V was identified as a positive effector of LPL activity (8–10).

ApoA-V has readily become recognized as an important determinant of plasma TG levels in humans and mice since its discovery 5 years ago (11, 12). Animal experiments using different strategies of underexpression and overexpression indicated an inverse relationship between apoA5 gene expression and plasma TG (11, 13) [e.g., adenoviral expression of apoA5 in mice resulted in a dose-dependent reduction of plasma TG levels (9)]. In humans, genetic variation at the APOA5 locus has been associated with HTG (11, 14–17). Moreover, homozygosity for truncation-causing mutations (Q148X and Q139X) in the APOA5 gene was recently shown

Abbreviations: apoA-V, apolipoprotein AV; HTG, hypertriglyceridemia, hypertriglyceridemic; MAb, monoclonal antibody; TC, total cholesterol; TG, triglyceride.

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to be associated with severe HTG in humans (18, 19). Hence, besides the deficiency of either LPL or apoC-II, mutations in the APOA5 gene were identified as a novel cause of type I hyperlipidemia (OMIM 238600), although it may be noted that patients with apoA-V mutations primarily present with type V hyperlipidemia (OMIM 144650).

Although the underlying mechanism is still obscure, several lines of evidence indicate that apoA-V stimulates LPL activity (8–10, 20–22). Whether this effect is direct, as suggested by dose-dependent stimulation of LPL activity in vitro (9), or indirect, through improved capturing of TG-rich lipoproteins by heparan sulfate proteoglycan-bound LPL (8, 22) or through stabilization of the active dimeric form of LPL, remains to be determined. It is of interest that apoA-V and apoC-III appear to have opposite modes of action (7). Accordingly, although gene disruption or overexpression of either apoA5 or apoC3 has a large impact on plasma TG levels, simultaneous deletion of both endogenous genes or simultaneous transgenic overexpression of human APOA5 and APOC3 has no effect on plasma TG levels in mice (23). In agreement with this, recombinant apoA-V was able to fully overcome the LPL-inhibitory effect of apoC-III in vitro (9). The relative amounts of apoA-V and apoC-III in plasma, and most likely their distribution over lipoproteins, may influence LPL activity and ultimately TG levels.

With the recent development of immunoassays for the quantification of apoA-V, it became apparent that plasma levels of apoA-V in normolipidemic subjects are rather low (157–198 ng/ml) compared with other apolipoproteins (24, 25). In this study, we aimed to determine apoA-V levels and study the correlation with lipid parameters in human subjects with varying degrees of HTG. Plasma apoA-V levels were determined using a newly developed ELISA.

**MATERIALS AND METHODS**

**Cohort**

Patients referred to our lipid clinic (Academic Medical Center, Amsterdam, The Netherlands) were recruited for this study cohort when fasting plasma TG levels were >10 mmol/l on the initial consultation. Subjects with mutations in the LPL gene, as determined by LPL gene sequencing, were excluded from the cohort. The studies conducted here were of European ethnicity and presented with type V hyperlipidemia. At the time of blood sampling for the current lipid and apolipoprotein measurements, the majority of the subjects (18 of 26) were using various lipid-lowering regimens (pharmaceutical or dietary intervention) to manage their hyperlipidemia. Fasted plasma obtained from normolipidemic volunteers (n = 42) was used to obtain reference values for apoA-V levels. The study protocol was approved by the institutional review board of the Academic Medical Center, and all participants gave written informed consent.

**Analytical procedures**

Fasting blood was collected in heparinized tubes. After centrifugation (15 min, 3,000 g at 4°C), the plasma was divided into aliquots and frozen at −80°C for later use; buffy coat was used for the isolation of genomic DNA. Plasma levels of TG and total cholesterol (TC) were measured using established enzymatic assays. Immunoblot detection of apoA-V in human plasma has been described previously (26). Heterozygotic hyperlipemic (HTG) subjects were genotyped for the −1131T>C and c.56C>G APOA5 polymorphisms as detailed elsewhere (17).

**Determination of plasma apoA-V levels by ELISA**

Plasma levels of apoA-V were determined using a newly developed sandwich ELISA. Anti-human apoA-V monoclonal antibodies (MAbs) B2B and E8E were obtained by genetic immunization of mice followed by boosting of animals with recombinant human apoA-V protein as described elsewhere (24). Different blocking agents were tested for their efficacy: it was noted that use of 3.0% BSA, 1.0% gelatin, or 1.0% nonfat dry milk powder as a blocking agent resulted in a high level (50–90%) of nonspecific binding of apoA-V to the microtiter plate (i.e., binding independent of the presence of capture MAb). Far better results were obtained when using 1.0% casein as a blocking agent, giving only 5% nonspecific binding.

Ninety-six-well microtiter plates (MaxiSorb; NUNC) were coated overnight at 4°C with 100 μl of MAb B2B (1.5 μg/ml in 50 mmol/l carbonate buffer, pH 9.6). Except for this coating step, all further plate handling was performed at room temperature and incubations were performed on a rocking platform. Plates were covered with an adhesive foil to prevent evaporation. After coating with capture MAb, plates were washed once with PBX (PBS containing 0.1% Triton X-100) and blocked by incubating for 1 h with 200 μl/well PBX (PBX containing 1.0% casein; Hammarsten grade; Merck). The plates were subsequently washed once with PBX, and, after incubation with PBX, samples, reference sera, and standards (100 μl/well, diluted in PBX) were added. Antigen was captured during a 2 h incubation period, followed by four washes with PBX to remove unbound and/or nonspecifically bound proteins. Captured antigen was detected by adding 100 μl/well biotinylated MAb E8E (1.0 μg/ml in PBX), and plates were incubated for 2 h. Excess detector MAb was washed away with four rinses with PBX. Then, 100 μl/well streptavidin-conjugated HRP (Dako) diluted 1:3,000 in PBX was added. After 30 min of incubation, plates were washed four times with PBX and 100 μl/well freshly prepared HRP substrate solution (100 μg/ml tetramethylbenzidine in 0.1 mol/l NaAc, pH 5.5, containing 0.003% hydrogen peroxide) was added. Product formation was stopped after exactly 30 min by the addition of 2 mol/l sulfuric acid (100 μl/well). After brief rinsing, absorbance at 450 nm was measured (Easia reader; Medgenix Diagnostics). Dilutions of a calibrated control plasma and recombinant human apoA-V (9) were included in each assay. Recombinant human apoA-V was >95% pure as estimated by SDS-PAGE, and recombinant protein concentration was determined by the bicinchoninic acid method using BSA as a standard. Calibration curves were fitted by linear regression, and correlation coefficients were typically >0.999. Reagent blanks had a typical absorbance of 0.060. Because of the nonlinearity of absorbance measurements, optical density values >2.0 were ignored and samples were reanalyzed at higher dilution.

**Determination of plasma apoC-III levels**

ApoC-III levels were determined using a sandwich ELISA specific for human apoC-III. Plasma samples were diluted in wash buffer (PBS and 0.05% Tween-20) containing 0.1% casein. Briefly, wells of Costar medium binding microtiter plates were coated overnight at 4°C with 100 μl of a polyclonal rabbit anti-human apoC-III antibody (1.0 μg/ml in PBS; Academy Biomedical Co., Houston, TX). After rinsing and the addition of samples, plates were incubated for 2 h at 37°C. After washing, HRP-conjugated goat
anti-human apoC-III polyclonal antibody (0.75 μg/ml in sample dilution buffer; Academy Biomedical Co.) was added. After incubation for 2 h at 37°C, HRP activity was detected with tetramethylbenzidine for 20 min at room temperature. Plasma from apoc3 wild-type mice spiked with human apoC-III (Academy Biomedical Co.) was used as a standard.

Statistical analysis

Date are presented as means ± SD. Pearson’s correlation coefficients and corresponding P values were calculated to assess the relationship between tested parameters. Mann-Whitney testing was used for comparisons between HTG subjects and normolipidemic controls. P < 0.05 was considered significant.

RESULTS

Characteristics of the apoA-V ELISA

For the determination of apoA-V levels, a sandwich ELISA was developed that used different MAbs for antigen capture (Mab B2B) and antigen detection (Mab E8E). For reference, we used plasma from the institutional blood bank in each assay. Serial dilutions of the reference plasma resulted in a typical sigmoid-shaped response curve (Fig. 1). The reference plasma was calibrated using recombinant human apoA-V of high purity (9) and contained 205.4 ± 12.5 ng apoA-V/ml. Semic quantitative analysis by immunoblotting was used as an independent method to validate apoA-V levels as determined by ELISA (Fig. 2A). Spiking of diluted reference plasma with known amounts (0.1–3.0 ng) of recombinant apoA-V resulted in calculated recoveries of 93.1–100.3%. The lower limit of detection of our ELISA was ~0.2 ng/ml. Intra-assay and interassay (n = 8–12) variations of three plasma samples (108–266 ng apoA-V/ml) were 2.4 ± 2.4% and 6.5 ± 2.3%, respectively.

Plasma apoA-V levels in HTG subjects

Fasted plasma apoA-V levels were determined in 28 HTG patients and in 42 normolipidemic controls. Relevant characteristics and biochemical parameters of these study groups are shown in Table 1. TG levels of the HTG individuals averaged 16.2 ± 18.7 mmol/l (range, 1.8–78.7 mmol/l). In addition, this cohort was characterized by increased TC levels compared with normolipidemic controls (7.6 ± 5.2 vs. 4.5 ± 0.9 mmol/l, respectively).

Importantly, we identified a marked 7.7-fold increase in mean plasma apoA-V levels in HTG subjects compared with controls (1,987 ± 2,603 vs. 258 ± 146 ng/ml, respectively; P < 0.001). Importantly, no interference was observed in our ELISA upon spiking of plasma samples with Intralipid to achieve a final TG level of 1.0 mmol/l, which is well above the final TG level of 0.035 mmol/l attained in diluted plasma of the most HTG subject (data not shown). The increased apoA-V level in the HTG group was confirmed qualitatively by immunoblot analysis using antibodies different from those used for ELISA (Fig. 2B). In addition, we noted on average 4.3-fold higher apoC-III levels in HTG subjects compared with controls (45.8 ± 33.8 vs. 10.6 ± 3.6 ng/dl, respectively; P < 0.001).

In agreement with published observations (17), the rare allele frequencies of two APOA5 variants that have been associated with increased TG levels appeared to be higher.

Fig. 1. Dose-response curves of native and recombinant apolipoprotein A-V (apoA-V) assayed by ELISA. Multiple dilutions of a reference plasma (open circles) and recombinant apoA-V standard (closed triangles) were assayed for apoA-V by ELISA as described in Materials and Methods. Values are depicted as means of duplicate measurements. The concentration range in which the assay was performed was 0.3–10.0 ng apoA-V/ml.

Fig. 2. Immunoblot validation of apoA-V levels determined by sandwich ELISA. Protein was separated on 10% polyacrylamide gels and transferred to polyvinylidene difluoride membranes. apoA-V was detected using a polyclonal anti-human apoA-V IgG fraction and appropriate secondary antibodies. A: Immunoblot analysis of human apoA-V (right four lanes; ELISA value = 4,035 ng/ml) and the indicated amounts of recombinant human apoA-V (right four lanes). Quantification of chemiluminescent signals led to a mean calculated value of 4,241 ng/ml for the plasma sample, validating the quantification by ELISA. B: Confirmation of increased apoA-V levels in hypertriglyceridemic (HTG) subjects using immunoblot analysis. Plasma apoA-V expression in a normolipidemic volunteer with an average apoA-V level (left lane; 205 ng apoA-V/ml) and five HTG subjects (right five lanes; 405, 826, 2,044, 3,935, and 7,767 ng apoA-V/ml, respectively) was detected using a polyclonal anti-human apoA-V IgG fraction and relative values derived from the quantification of chemiluminescent apoA-V signals.
in the HTG group (0.23 and 0.17 for the −1131T>C and c.56C>G polymorphisms, respectively).

Correlates of apoA-V levels with lipid and apoC-III levels

In the HTG subjects, we identified a weak but statistically significant positive correlation between plasma apoA-V levels and TG (ρ = +0.44, P = 0.02) (Table 2, Fig. 3A). As evident from Fig. 3A, a number of HTG subjects had extremely high apoA-V levels. Replicate analysis (at several dilutions) of apoA-V/TG levels in the respective plasma samples, however, indicated that these measurements were highly reproducible. Moreover, APOA5 gene sequencing revealed that in only a single subject of this cohort might the observed increase in apoA-V level have been attributable to the accumulation of a mutant apoA-V protein (data not shown). From Fig. 3A, it can be appreciated that the observed correlation is highly dependent on the extreme apoA-V values in some patients in this small cohort. Therefore, we performed a series of analyses excluding data from one or more of these patients. Omission of single or multiple extreme TG/apoA-V values (using >50 mmol TG/l and >5,000 ng apoA-V/ml as arbitrary cutoff points) still provided evidence of a positive correlation between apoA-V and TG levels with ρ values ranging from +0.07 to +0.68 (data not shown). Separate analysis of less extreme ranges of TG/apoA-V levels also revealed positive and significant correlations between apoA-V and TG levels (e.g., ρ = +0.61, P = 0.04 in subjects having <10.0 mmol TG/l and <1,500 ng apoA-V/ml). In normolipidemic subjects, a very weak positive correlation between apoA-V and TG did not reach statistical significance (ρ = +0.11, P = 0.47) (Table 2, Fig. 3E).

ApoC-III levels showed positive and significant correlations with TG and TC in both the HTG and normolipidemic groups (Table 2). Of interest is the strong positive correlation (ρ = +0.66, P < 0.001) (Fig. 3C) between apoA-V and apoC-III levels in the HTG group; this correlation did not reach significance in the normolipidemic group (ρ = +0.24, P = 0.12) (Fig. 3G). In view of the increase in both apoA-V and apoC-III levels in the HTG group, it could be argued that the positive correlation between TG and apoA-V levels was confounded by apoC-III. Indeed, when controlling for apoC-III levels, no correlation between apoA-V and TG was apparent in the HTG group (partial ρ = +0.09, P = 0.67). In contrast, apoC-III and TG remained positively correlated in both the HTG and control groups when controlling for apoA-V (partial ρ = +0.43, P = 0.025 and partial ρ = +0.78, P < 0.001, respectively).

ApoA-V did not correlate with TC in the HTG group (ρ = +0.09, P = 0.66) (Fig. 3B), but did show significant positive correlation with TC in the normolipidemic group (ρ = +0.44, P = 0.004) (Fig. 3F), independent of apoC-III (data not shown).

Correlation of the molar ratio of apoC-III over apoA-V and TG levels

It is noteworthy that apoC-III is far more abundantly present in plasma than its counterpart apoA-V. Therefore, we tested whether the molar ratio of apoC-III over apoA-V, which may represent a measure for the balance between LPL-inhibitory and -stimulatory activities, correlates with TG levels. Bearing in mind that the quantitative distribution of these apolipoproteins over the various lipoproteins is unknown and may be relevant in

| TABLE 2. Pearson correlation coefficients (r) and two-tailed P-values of the indicated parameters in HTG and normolipidemic subjects |
|---------------------------------------------------------------|
| **Parameter** | **HTG Subjects** | **Normolipidemic Subjects** |
|----------------|------------------|----------------------------|
| ApoA-V and TG | +0.44  | 0.02   | +0.11  | 0.47  |
| ApoC-III and TG | +0.58  | 0.001  | +0.78  | <0.001 |
| ApoC-III/apoA-V ratio and TG | +0.19  | 0.32   | +0.37  | 0.015 |
| ApoA-V and apoC-III | +0.66  | <0.001 | +0.24  | 0.12  |
| ApoA-V and TC | +0.09  | 0.66   | +0.44  | 0.004 |
| ApoC-III and TC | +0.54  | 0.004  | +0.36  | 0.02  |
this regard, we did not find evidence for such a corre-
orrelation in the HTG group ($r = 0.19$, $P = 0.32$) (Fig. 3D).

In normolipidemic subjects, however, we identified a
positive correlation ($r = 0.37$, $P = 0.015$) (Fig. 3H).

Of note, the average apoC-III/apoA-V molar ratio was not
different in HTG and normolipidemic subjects (2.3 ± 1.5 vs. 2.4 ± 1.5, respectively; $P = 0.074$).

**DISCUSSION**

Using a newly developed ELISA, we demonstrate that
apoA-V levels are markedly increased in patients with
HTG. This finding was confirmed with different antibi-
dies in an immunoblot analysis. In the same group of
patients, apoC-III levels were also increased. We identified
a positive correlation between apoA-V and TG in this
group of patients; interestingly, however, this relation was
abolished after correcting for apoC-III levels.

Including the current description, four ELISA methods
have now been described for the quantitative analysis of
apoA-V levels in humans. Despite a multitude of poten-
tially confounding factors in the various study groups (e.g.,
nethnicity, gender, age) and differences in assay design, the
values reported for the respective control populations are
generally in good agreement [i.e., 198 ng/ml (24), 157 ng/
ml (25), 258 ng/ml (this study)], although the values re-
ported by Pruneta-Deloche et al. (27) appear markedly
lower (i.e., 10 ng/ml). The positive correlation between
apoA-V and TG observed in the present study, however,
contrasts with findings by others. First, O’Brien et al. (25)
described a negative correlation between apoA-V and TG
levels in 40 normolipidemic volunteers (albeit in the ab-
sence of information on statistical significance and correla-
tion coefficient). Subsequently, a weak negative correla-
tion between plasma apoA-V and TG levels ($r = -0.22$, $P =
0.021$) was noted in normolipidemic female but not male
subjects (24). Moreover, the latter study indicated lower
plasma apoA-V levels in HTG type II diabetic subjects and in
a mixed-gender population of carriers of the rare allele of
the $-1131T>C A P O A 5$ polymorphism that is associated
with HTG (24).

Although the above observations are in support of an
inverse relationship between apoA-V and TG levels, the
present findings and those of others indicate that this rela-
tionship may be more complex, both in humans and in
rodents (28). Specifically, Pruneta-Deloche et al. (27) ob-
served increased postprandial plasma apoA-V and TG levels
in patients with type II diabetes. Furthermore, Becker et al.
(29) observed an increase of both plasma apoA-V and TG
levels in subjects recovering from sepsis. Concordantly, our
study shows a marked increase of plasma apoA-V levels in
HTG subjects. Importantly, the concept that apoA-V and TG
are indeed positively related is supported by very recent
findings by Dallinga-Thie et al. (30), Vaessen et al. (31), and
Henneman et al. (32) in patients with type II diabetes,
apparently healthy individuals, and patients with severe
HTG, respectively.

Given the complexity of the regulation of plasma TG
levels, correlations between individual factors and TG may
be confounded by other factors, as exemplified by the
The absence of a correlation between apoA-V and TG after adjusting for apoC-III levels (30; this study). Detailed knowledge of all of the factors involved in the regulation of TG levels and quantitative assessment of their levels in plasma will ultimately be helpful in establishing the relative importance of individual factors.

Multiple factors may have contributed to the increased apoA-V levels in the patient group, including medication and diabetes. For example, among medication taken by the subjects in the current HTG group, lipid-lowering fibrates have been shown to upregulate \( \text{apoA-V} \) mRNA levels in vitro (33, 34). A different type of peroxisome proliferator-activated receptor \( \alpha \) agonist has been shown to increase plasma apoA-V levels in cynomolgus monkeys after 3 days of administration, apparently lagging behind the compounds’ earlier TG-lowering action (35). Nevertheless, one-way ANOVA identified only diabetes as a significant contributor to both increased apoA-V (\( P = 0.030 \)) and apoC-III (\( P = 0.032 \)) levels in our cohort of HTG subjects, with no significant effects of medication, the \(-1111\text{TT/CC} \) and \( c.56\text{CC/GG} \) polymorphisms (Table 3), gender, age (lower vs. upper half), body mass index (<25 vs. \( \geq 25 \)), or alcohol consumption (no intake vs. alcoholic intake) (data not shown). Importantly, excluding the patients with diabetes and those taking medication, apoA-V levels were still high (i.e., >95th percentile of the normolipidemic group) in four of eight HTG subjects.

In summary, apoA-V has a potent lipid-lowering action in experimental animals, which has led to the idea that plasma apoA-V levels would correlate negatively with plasma TG levels in humans. The current data, in conjunction with recently published reports (30–32), however, do not support such a relationship. In this study, we specifically observed marked increases of both apoA-V and apoC-III levels in HTG subjects. When adjusted for apoC-III levels, no independent correlation between apoA-V and TG was apparent. In agreement with the intricacy of the regulation of the catalytic activity of LPL, dimers bound to the vessel wall, the correlation of apoA-V and TG levels is not as straightforward as previously anticipated and requires additional factors to be considered.

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