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Plasma apoAV levels are markedly elevated in severe hypertriglyceridemia and positively correlated with the APOA5 S19W polymorphism

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ABSTRACT

Objective: The recently discovered apoAV is hypothesized to affect triglyceride metabolism by stimulating the lipolysis of triglycerides in VLDL and chylomicrons. We set out to determine the association between increased serum TG levels, plasma apoAV levels, and polymorphism of the APOA5 gene, with specific emphasis on the APOA5 S19W variation. This mutation alters the endoplasmic reticulum signal peptide and is hypothesized to impair apoAV secretion into the circulation.

Methods and Results: Two haplotype-tagging APOA5 polymorphisms, APOA5 S19W and APOA5 -1131T>C and plasma apoAV levels were determined in a previously characterized population of patients with severe hypertriglyceridemia (HTG). As compared to a random control population, the allele frequencies of the APOA5 S19W and -1131T>C rare variants were significantly increased in HTG patients. Furthermore, the HTG population exhibited markedly elevated plasma apoAV levels that were positively correlated with serum TG levels. Plasma apoAV levels were positively correlated with occurrence of the APOA5 S19W rare variant.

Conclusions: The increased allele frequencies of the APOA5 S19W and -1131T>C rare variants in the HTG population are in agreement with previous reports. Our data show a positive correlation between apoAV and TG levels. Moreover the finding of a positive association between apoAV levels and the APOA5 S19W rare variant is in disagreement with the hypothesis that this variant is poorly secreted.

INTRODUCTION

The recently identified apolipoprotein (apo) AV^{1,2} has been shown to affect triglyceride (TG) levels in mouse models. Serum TG concentrations in mice were 4-fold increased upon deficiency of the endogenous APOA5 gene and were decreased by 65% by overexpression of the human APOA5 gene¹. Adenovirus-mediated transfer of murine APOA5 to mice resulted in a dose-dependent reduction of serum TG^{3,4}. Thus, in these animal model studies, plasma apoAV levels are negatively correlated with serum TG levels.

In a large number of human populations, polymorphisms in the APOA5 gene have been associated with variation in serum TG level (for reviews see⁵⁻⁷). Moreover, deficiency for apoAV has been associated with HTG⁸⁻¹⁰, confirming the TG-lowering activity of apoAV. The APOA5 gene is localized in a gene cluster containing APOC3, APOA1 and APOA4, characterized by a high level of linkage disequilibrium. Single nucleotide polymorphisms (SNPs) in the APOA5 gene fall into three common haplotypes: APOA5*1, with common alleles at all sites; APOA5*2, with rare alleles of -1131T>C, -3A>G, 751G>T, and 1891T>C; and APOA5*3, distinguished by c.56C>G (S19W)^{11,12}.

ApoAV is a 39 kDa protein (343 amino acids) that may enhance LPL mediated TG hydrolysis, most likely in concert with heparin sulphate proteoglycans^{3,13,14}. The APOA5 S19W mutation is located in the endoplasmic reticulum signal peptide and is hypothesized to decrease the apoAV secretion rate¹. Recent studies using a secreted alkaline phosphatase as reporter protein for signal peptide function are in agreement with this hypothesis¹⁵.

In the current study, we have screened a severely hypertriglyceridemic population for the frequency of the most common APOA5 haplotype tagging SNP's, -1131T>C for APOA5*2 and S19W for APOA5*3. In addition, we have determined the plasma apoAV levels and correlated these levels with serum TG levels and APOA5 haplotype tagging SNPs. We find an increased frequency of the APOA5 -1131C>T

and APOA5 S19W rare variants in the HTG population and markedly elevated plasma apoAV levels. Moreover, the apoAV levels are positively associated with serum TG levels. These data do not support the previously reported negative association between plasma apoAV and TG levels^{16,17} and presumed secretion defect of the APOA5 S19W rare variant.

MATERIALS AND METHODS

PATIENT AND CONTROL POPULATION RECRUITMENT

Hypertriglyceridemic subjects were recruited from our lipid clinic meeting the following criteria: serum triglyceride >3.8 mmol/L, VLDL-cholesterol >1 mmol/L, LDL-cholesterol < 4.5 mmol/L and no apoE2/E2 phenotype^{18,19}. The diagnosis was based on the means of two fasting blood samples obtained after a step one diet of the NCEP for at least 8 weeks. Patients with renal-, liver- or thyroid disease and/or alcohol consumption >40g/day were excluded. 29 (21%, N=139) HTG patients had type II diabetes. The random control panel include a total of 175 anonymous subjects. Data on age, body mass index and lipid levels of random control subjects were not available. The random control population consisted of 100 anonymous blood donors, blood supplied by Sanquin (Leiden, the Netherlands) and 75 anonymous subjects of a population-based study among 2018 randomly selected 35-year-old men²⁰ (Table 1). Informed consent was given by each participant and the study was approved by the Medical Ethics Committee of the LUMC. All blood samples were collected after an overnight fast. Serum was obtained after centrifugation at 1500 g for 15 min at room temperature. Lipids and lipoproteins were measured after ultracentrifugation using standard methods²¹.

GENETIC ANALYSIS

Identification of the polymorphisms was performed using PCR followed by restriction enzyme analysis. The following SNPs were analyzed: APOA5 c.56 C>G (S19W) and APOA5 -1131T>C (SNP3)^{12,22}. For amplification of the APOA5 S19W fragment (c.56 C>G) the following primers were used: 5'-CCA GAA GCC TTT CCG TGC CTG GGC GGC-3' (sense) and 5'-TGT AAA ACG GCC AGT - AAA AGG AAA A-CGG CCG-GTG CTC ACC TGG GCT GCT CTT C-3' (antisense). The penultimate base in the forward primer (sense) was changed to a c to create a Eco521 recognition site in the common allele. This recognition site was also introduced in the reverse primer (antisense), which served as a digestion control. To establish an optimal visualization, the 3' primer also was elongated with a 5' additional M13 tail. Fragments of 24, 156 and 27 bp represented the Ser-19 allele and the fragments 24 and 184 bp represented the Trp-19 allele.

The primers for amplification of the APOA5 -1131T>C SNP were as follows: 5'-CCC CAG GAA CTG GAG CGA AAT T-3' (sense) and 5'-TGT AAA ACG GCC AGT AAA AGG AAA AGG AAA AGG TTA AGA TTG ATT CAA GAT GCA TTT AGG AC-3' (anti-sense). The penultimate base in the forward primer (sense) was changed to a T to create a Tru1 recognition site in the common allele. This recognition site was also introduced in the reverse (antisense) primer, which served as a digestion control. To establish an optimal visualization, the 3' primer also was elongated with a 5' additional M13 tail. Fragments of 21, 168 and 36 bp represented the -1131T allele and the fragments 36 and 189 bp represented the -1131C allele.

ANALYSIS OF PLASMA APOAV LEVELS

Total plasma apoAV levels were measured by means of a sandwich ELISA assay²³. A subset of the population, representing individuals from all tertiles with respect to serum TG and apoAV levels (as determined by ELISA) was analyzed by semi-quantitative Western blot analyses as previously described².

STATISTICAL ANALYSIS

For both polymorphisms and haplotypes, the Hardy-Weiberg equilibrium was calculated using the gene-counting method and differences were assessed by chi-squared analysis. *P*-values lower than 0.05 were considered as significant differences. Differences in sex were evaluated with Chi-squared analysis. Data in table 1 are expressed as mean \pm SD. Data in figure 1,2 and 3 are expressed as mean \pm S.E.M. Since total TG and apoAV levels showed non-Gaussian distributions, the mean differences of these parameters between groups were calculated by means of Mann-Whitney analysis. Association analysis between the TG concentration and apoAV concentration was performed by subdividing TG levels in quartiles (3.80-6.31, 6.53-9.42, 9.45-15.71 and 16.64-82.14 mmol/L resp.) All statistical analyses were performed with SPSS for windows 11.0.1 (SPSS, Chicago, IL, release November 2001).

RESULTS

FREQUENCY OF APOA5 HAPLOTYPE-TAGGING SNP'S IN HTG AND CONTROL POPULATION

The occurrence of 2 common haplotype-tagging APOA5 SNPs, APOA5 -1131 T>C (haplotype APOA5*2) and APOA5 c.56C>G (S19W, haplotype APOA5*3) were determined in 141 patients with severe HTG and 175 control individuals (random control panel) (for population characteristics, see Table 1).

Table 1: Characteristics of patients with hypertriglyceridemia and controls

	HTG	N	RCP	N
Male	119 (84.4 %)	141	171 (97.7 %)	175
Female	22 (15.6 %)	141	4 (2.3 %)	175
Age	53.9 \pm 10.7	141		
range	26-78			
BMI (kg/m ²)	27.7 \pm 3.4	141		
Total triglycerides (mmol/L)	14.3 \pm 13.8	141		
VLDL-triglycerides (mmol/L)	12.0 \pm 12.3	132		
apoAV (ng/ml)	965.2 \pm 1391.8	129		
Total cholesterol (mmol/L)	8.8 \pm 3.9	141		
HDL-C (mmol/L)	0.68 \pm 0.20	137		
VLDL-C (mmol/L)	5.0 \pm 4.1	132		
LDL-C (mmol/L)	2.8 \pm 1.5	132		
HOMA index	14.0 \pm 16.7	135		

RCP indicates random control panel; N indicates total number of subjects with available data about respective parameter; Age, body mass index, lipid levels, HOMA index and apoAV levels are presented as mean \pm SD

Allele frequencies for both polymorphisms in the HTG and random control subjects were in Hardy-Weinberg equilibrium. Allele frequency of the rare APOA5 -1131 T>C SNP is 5.9% in the control population versus 23.5% in the HTG population (*P*<0.05). Similarly, allele frequency of the rare variant of the APOA5 S19W is 4.4 % in the control population versus 19.1 % in the HTG population (*P*<0.05). Thus, both the APOA5 -1131T>C and APOA5 S19W rare variants are significantly increased in the HTG population.

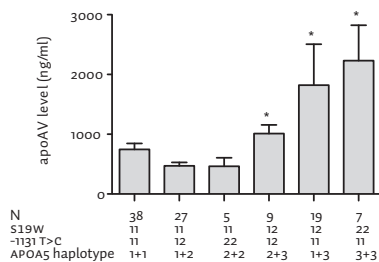


Fig.1. Association between the APOA5 S19W and APOA5 -1131T>C polymorphisms and plasma apoAV level in HTG patients. ApoAV concentration is expressed as mean \pm s.e.m. Data are shown for all individuals characterized for the parameters involved. Differences between non-carriers and carriers of the rare variants were evaluated with Mann-Whitney analysis. * $P < 0.05$ as compared with S19W 11/-1131 T>C 11; S19W 11/-1131 T>C 12; S19W 11/-1131 T>C 22. †Haplotype definitions as described by others^{6,12,26,28}.

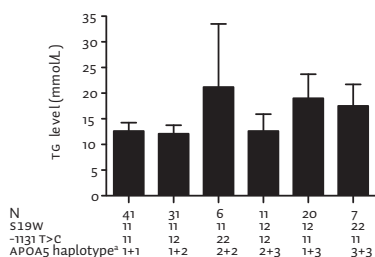


Fig.2. Association between the APOA5 S19W and APOA5 -1131T>C polymorphisms and serum TG level in HTG patients. Triglyceride concentration is expressed as mean \pm s.e.m. Data are shown for all individuals characterized for the parameters involved. Differences between non-carriers and carriers of the rare variants were evaluated with Mann-Whitney analysis. †Haplotype definitions as described previously^{6,12,26,28}.

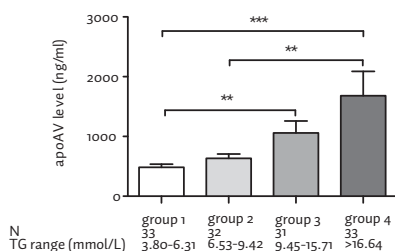


Fig.3. Association between the serum TG concentration and plasma apoAV concentration in HTG patients. The HTG population was divided in quartiles based on TG concentration. ApoAV levels are expressed as mean \pm s.e.m. Data are shown for all individuals characterized for the parameters involved. Differences between groups were evaluated with Mann-Whitney analysis. Significant differences are expressed as: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

ASSOCIATION BETWEEN PLASMA APOAV, TG LEVELS AND APOA5 POLYMORPHISMS

Plasma apoAV levels in the HTG population were determined by sandwich ELISA. ApoAV levels in a selected set of individual sera were confirmed by an independent ELISA and also by semi-quantitative Western Blot analyses (data not shown). All replicate data were in near perfect concordance. Average plasma apoAV concentration in the HTG population was 0.97 ± 1.39 μ g/ml, which is 4 to 5-fold increased as compared to reported apoAV levels in normal individuals^{16,17,23}.

Twenty HTG patients were taking antihypertensive medication. The apoAV levels were not significantly different between the treated and untreated groups of patients. Association studies were performed between APOA5 polymorphic variants and plasma apoAV and TG levels. Figure 1 shows that there is a significant positive correlation between presence of the APOA5 S19W rare variant and plasma APOA5 levels ($P < 0.05$). No association was found between presence of the APOA5 -1131T>C rare variant and plasma APOA5 levels.

Neither the APOA5 S19W nor the APOA5 -1131T>C rare variants were correlated with serum TG levels (Fig. 2). The plasma apoAV levels were positively correlated with serum TG levels. The apoAV level in the lowest quartile of serum TG level (up to 6.31 mmol/L) is significantly different from the apoAV levels in all subsequent quartiles of serum TG level (Fig. 3).

DISCUSSION

Here, we show that the frequencies of the APOA5 S19W and -1131T>C variant allele carriers are about four times higher in patients with severe hypertriglyceridemia than in the control population, indicative for a causal association of the APOA1/C3/A4/A5 locus with this complex lipid disorder. Plasma apoAV levels are approximately 5-fold increased as compared to normal controls¹⁶ and positively correlated with serum TG levels and the APOA5 S19W rare variant. The increased frequencies of the APOA5 S19W and -1131T>C rare variants in the HTG population are in line with previous reports, that have demonstrated a positive correlation between both these variants and TG levels in

various populations²⁴⁻²⁷ (for reviews see⁵⁻⁷). Somewhat surprisingly, within the HTG population itself, the correlation between TG levels and presence of the *APOA5* S19W or *APOA5* -1131C>T rare variants was not significant (Fig. 2). However, since a trend seems present, this may be a power problem due to the size of the population. In addition, the lack of correlation may be caused by the selection criteria used for inclusion of patients in the HTG population. Half of the HTG population is characterized by TG levels of more than 9 mmol/L (upper two quartiles of the population (Fig. 3). It is more than likely that especially these quite extreme HTG patients will have mutations in additional genes causing the high TG levels that will be dominant over the effects associated with the *APOA5* variants.

The increased average apoAV levels in the presence of HTG and the positive correlation between apoAV levels and TG levels indicate that, at least in these patients, the presumed LPL-stimulatory activity of apoAV does not normalize circulating TG levels. This observation contrasts with a study in Japanese individuals that demonstrated a negative correlation between plasma apoAV and serum TG levels¹⁶ and with two other independent studies which both found that apoAV deficiency is associated with HTG in humans^{8,9}. Interestingly, individuals that are heterozygous for apoAV deficiency have a highly variable phenotype^{8,9}, indicating that additional factors may be necessary for the expression of apoAV-associated hyperlipidemia. The observation that the phenotypes of apoAV overexpression and apoAV-deficiency in mice are so clear cut, could indicate that these factors are skewed in a species-specific manner as to emphasize the role of apoAV. The identity and role of these putative modifiers remain to be established.

Several explanations could be invoked to explain the positive correlation between apoAV and TG levels and some of these may be affected by species-specific modifiers. *First*, the disturbance in TG metabolism in a large fraction of the HTG patients is dominant over the presumed apoAV TG-lowering effect. For example, if more potent inhibitors of lipolysis are up-regulated. It is possible that apoAV may actually even be up-regulated as a consequence of the HTG, but to no avail. Analysis of the production and turn-over rate of apoAV should clarify this issue. *Second*, the increased plasma levels of apoAV are a consequence of the altered lipoprotein structure present in HTG. If apoAV has for example a higher affinity for large-sized TG-rich particles, the observed increase in plasma apoAV levels is a symptom of HTG²³. *Third*, apoAV could be up-regulated as an indirect consequence of HTG. It is conceivable that apoAV may have an as yet unknown function in liver homeostasis. For example, it is unknown what the mechanism is underlying the up-regulation of *APOA5* expression after partial hepatectomy². It should be noted that plasma apoAV levels did not correlate with SAA levels and thus presumably not with liver dysfunction per se (data not shown). Independent of the explanation underlying the increased apoAV levels in HTG patients, it is obvious that in the majority of individuals of this HTG population, the TG-lowering effect of apoAV is not sufficient to normalize TG levels.

The analyses of *APOA5* variant allele frequencies and correlations with apoAV and TG level were performed under the assumption that the *APOA5* S19W and *APOA5* -1131T>C rare variants are allelic-tagging variants. Although we have not performed a haplotype analysis in the HTG population, this assumption seems reasonable since the *APOA5* haplotypes are in Hardy-Weinberg equilibrium in the HTG population. Moreover, the haplotype structure has been confirmed in numerous population studies^{6,12,26,28}. Since we have not studied other polymorphic sites in *APOA1/C3/A4/A5* locus, we cannot draw conclusions on the causality between the presence of the *APOA5* S19W and *APOA5* -1131T>C rare variants and HTG or apoAV level. However, it is of interest to note that the *APOA5* S19W variant allele is characterized by presence of the most common (wild type) variants of other SNP's in the *APOA1/C3/A4/A5* locus^{12,26,28}. The *APOA5* S19W variation occurs in the signal peptide for co-translational transport

into the endoplasmic reticulum. Computer modeling of this variation indicated a conformational change and *in vitro* analysis using a secreted alkaline phosphatase as reporter demonstrated that the variant signal peptide resulted in 50% less efficient secretion as compared to the wild type form¹⁵. Therefore, the positive correlation between the presence of the APOA5 S19W rare allele and serum apoAV levels is unexpected.

This study was designed to test the hypothesis that apoAV levels are negatively correlated with HTG. The positive correlation between plasma apoAV levels and TG levels on the one hand and apoAV levels and the APOA5 S19W rare variant on the other hand provide evidence against (1) a dominant role for apoAV in reducing serum TG levels in HTG patients and, (2) a secretion defect associated with the APOA5 S19W variant. These observations illustrate that the postulated role of apoAV as dominant gene determining serum TG levels in humans may require adjustment.

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