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Objective—The genetic determinants of severe hypertriglyceridemia (HTG; MIM 144650) in adults are poorly defined. We therefore resequenced 3 candidate genes, namely *LPL*, *APOC2*, and *APOA5*, to search for accumulation of missense mutations in patients with severe HTG compared with normolipidemic subjects.

Methods and Results—We resequenced >2 million base pairs of genomic DNA from 110 nondiabetic patients with severe HTG and determined the prevalence of coding sequence variants compared with 472 age- and sex-matched normolipidemic controls. We found: (1) heterozygous mutations (*LPL* p.Q-12E >11X, p.D25H, p.W86R, p.G188E, p.I194T and p.P207L; *APOC2* p.K19T and IVS2–30G>A) in 10.0% of severe HTG patients compared with 0.2% of controls (carrier odds ratio [OR] 52, 95% confidence interval [CI] 8.6 to 319); and (2) an association of the *APOA5* p.S19W missense variant with severe HTG (carrier OR 5.5 95% CI 3.3 to 9.1). Furthermore, either rare mutations or the *APOA5* p.S19W variant were found in 41.8% of HTG subjects compared with 8.9% of controls (carrier OR 7.4, 95% CI 4.5 to 12.0). Also, heterozygotes for rare mutations had a significantly reduced plasma triglyceride response to fibrate monotherapy.

Conclusions—Both common and rare DNA variants in candidate genes were found in a substantial proportion of severe HTG patients. The findings underscore the value of candidate gene resequencing to understand the genetic contribution in complex lipoprotein and metabolic disorders. **(*Arterioscler Thromb Vasc Biol*. 2007;27:2450-2455.)**

Key Words: complex trait ■ metabolism ■ atherosclerosis ■ pancreatitis ■ mutation

Hypertriglyceridemia (HTG) is a commonly encountered phenotype that is a defining component of the metabolic syndrome[1] and is associated with numerous comorbidities, including increased coronary heart disease (CHD) risk.[2] Furthermore, plasma triglyceride (TG) concentrations >10 mmol/L—a level that defines adult patients with Frederickson type 5 hyperlipoproteinemia (MIM 144650)—are associated with increased risk of acute pancreatitis.[3,4] Plasma TG concentration >10 mmol/L is seen in ≈1 in 600 adult North Americans.[5] Although both genetic and lifestyle factors determine plasma TG concentration, the genetic component remains incompletely defined.[6]

Complex quantitative traits, such as plasma TG, do not conform to Mendelian inheritance patterns; instead their genetic basis represents the cumulative contribution of multiple DNA variants.[7] A promising new strategy in human genetics to understand common complex traits is called the "missense accumulation approach", which aims to detect enrichment of rare, deleterious missense DNA variants in cases taken from one extreme of the distribution of a quantitative trait versus a control group.[7] The cumulative frequency of missense mutations rather than their individual frequencies is then compared between cases and controls.[7] This method has proven to be successful for investigation of common disease traits that have a very heterogeneous spectrum of predisposing alleles.[7] For instance, the missense accumulation approach has been successfully used to evaluate the *MC4R* gene[8] and several other genes in obesity,[9] and the tyrosine phosphatome in colorectal cancers.[10]

However, the most successful application of the missense accumulation strategy has been in lipoprotein metabolism, as evidenced by the pioneering work of Hobbs and Cohen.[11–14] They have found enrichment of missense mutations in individuals at the extremes of several plasma lipoprotein traits, including: (1) increased missense mutations in *LCAT*, *APOA1*, and *ABCA1* among individuals with depressed high-density lipoprotein (HDL) cholesterol[13]; (2) increased *PCSK9* missense or nonsense mutations among individuals with depressed low-density lipoprotein (LDL) cholesterol[14]; (3) increased missense mutations in *NPC1L1* in individuals with reduced sterol absorption and low plasma LDL cholesterol[12]; and (4) increased missense mutations in *ANGPTL4* in indi-

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viduals with depressed triglyceride (TG) and increased HDL cholesterol.[11] In these studies, the statistical association of the accumulation of rare coding sequence variants implicated the gene as contributing to the traits under study, whereas no direct experimental evidence of dysfunction for the mutations was provided.[11–14] Furthermore, most rare missense variants that accumulate in patients clustered at the extremes of a quantitative trait are dysfunctional.[7]

Homozygous mutations in candidate genes for plasma TG metabolism, namely *LPL* encoding the main plasma hydrolytic enzyme lipoprotein lipase (LPL), and *APOC2* encoding its circulating cofactor apolipoprotein (apo) C-II, are found in patients with Frederickson type 1 hyperlipoproteinemia[15–17] (MIM 238600), a disorder that affects ≈1 in 1 million people.[13,15] Also, homozygous nonsense mutations in *APOA5* encoding apo A-V, a protein that promotes LPL activity,[18] have been found in probands with late-onset chylomicronemia.[19] Because the prevalence of coding sequence variants in adults with severe HTG is unknown, we resequenced a total of >2 million base pairs of genomic DNA from nondiabetic patients with severe HTG and used the missense accumulation approach to determine the association of variants in *LPL*, *APOC2*, and *APOA5* with severe hypertriglyceridemia.

Methods

Subjects

We studied 110 nondiabetic patients of European geographic ancestry with severe HTG, defined as having fasting plasma TG >10 mmol/L documented on ≥2 occasions, from a single tertiary referral lipid clinic. Patients underwent a complete medical history and examination; basic clinical, biochemical, and demographic variables were collected. Normolipidemic nondiabetic adult controls were taken from the European subgroup of the Study of Health Assessment and Risk in Ethnic groups (SHARE), a survey of cardiovascular risk factors in Canadian subpopulations[20] together with healthy population-based controls from the same region of Canada. No control had ischemic heart disease and there was no use of medications among these healthy control subjects. All patients provided informed consent for DNA analysis.

DNA Analysis

DNA was extracted as described.[21] Coding regions and intron-exon boundaries of *LPL* (10 exons), *APOC2* (4 exons), and *APOA5* (4 exons) were amplified, purified, and then directly sequenced in 5′- and 3′- directions in an ABI 3730 DNA Analyzer (Applied Biosystems) using reagents shown in supplemental Table I. DNA sequences were analyzed using Sequence Navigator software (Applied Biosystems). DNA variants were confirmed in an independent sample on another day. Screening of controls for sequence variants was performed using allele-specific methods such as restriction endonuclease analysis or a method called SNaPshot (Applied Biosystems), as summarized in supplemental Table II. Blinded between-day replicated genotypes of a random 5% of samples showed >99.9% concordance. DNA variants with a minor allele frequency (MAF) <1% in controls were analyzed separately from variants with MAF >1%.

Bioinformatic Studies

We used both the PANTHER (www.pantherdb.org)[22,23] and PolyPhen[24] algorithms to impute dysfunction of sequence variants. Predictions of dysfunction from both programs are well correlated with in vitro functional assessment.[24,25] The scores from each program were grouped into 3 categories: "probably deleterious", "possibly deleterious", and "benign". The majority of biochemically-proven functional mutations have scores of either "probably" or "possibly" deleterious for both programs.[24,25]

Table 1. Baseline Attributes of Study Subjects

	Severe HTG	Controls	*P* Value
No.	110	472	
Percent female	32.1%	37.3%	NS (0.29)
Age, years	49.9±12.9	47.3±14.4	NS (0.10)
Body mass index, kg/m^2	29.4±4.2	27.8±4.4	NS (0.67)
Total cholesterol, mmol/L	11.9±6.0	5.20±0.93	<0.0001
Triglycerides, mmol/L	32.6±26.5	1.46±0.73	<0.0001
HDL-cholesterol, mmol/L	0.78±0.35	1.23±0.33	<0.0001

Data shown for quantitative variable are means±SD.

HTG indicates hypertriglyceridemia; HDL, high-density lipoprotein; NS, not significant at nominal level of significance.

Statistical Analysis

Analyses were performed using SAS version 9.1 (SAS Institute). Between-group differences in discrete and quantitative traits were determined using chi-square analysis and unpaired Student *t* tests, respectively. Odds ratios (OR) were calculated using the "case-control" method in the FREQ procedure in SAS. Log transformed TG was used for parametric analyses, but untransformed values are shown in the tables and figure. The nominal level for significance was $P<0.05$.

Results

Clinical and Biochemical Features

Baseline attributes of the study sample are shown in Table 1. 110 nondiabetic severe HTG cases were each matched with up to 4 controls based on age within 5 years and sex. By definition, severe HTG patients had markedly higher plasma TG and total cholesterol and significantly lower HDL cholesterol. Plasma TG concentration in severe HTG patients ranged from 10.1 to 180 mmol/L. In addition, 32/110 severe HTG patients (29.0%) had been hospitalized on ≥1 occasion with pancreatitis.

Rare Mutations in Candidate Genes

Mutations, defined as DNA sequence variants with MAF <1% in controls or known functional disease-causing mutations that were found in the genomic DNA of severe HTG patients, with their highest recorded plasma TG concentrations, are summarized in Table 2. In the severe HTG patients, we found 12 occurrences of heterozygous candidate gene mutations. Carriers were heterozygous for 1 of 9, mostly known disease-causing, mutations: 6 in *LPL*, 2 in *APOC2* and 1 in *APOA5*. For instance, in the homozygous state, *LPL* p.W86R, p.G188E, p.I194T, and p.P207L each cause HLP type 1 and each is dysfunctional in vitro, with significantly impaired or absent hydrolytic capacity of the mutant gene product.[26–29] Among the novel heterozygous mutations observed in this study, *LPL* p.Q-12E >11X was a frameshift mutation with a very prematurely truncated product, whereas *LPL* p.D25H was predicted to be deleterious in both PANTHER and PolyPhen. The known *APOC2* p.K19T variant was previously associated with dyslipidemia.[30,31] The novel *APOC2* IVS2–30G>A variant potentially affects RNA splicing. *APOA5* p.A315V[32] was predicted to be possibly delete-

Table 2. Rare DNA Sequence Mutations in *LPL*, *APOC2*, and *APOA5* in Patients With Severe Hypertriglyceridemia

		Predicted Dysfunction		No. of Carriers		
Variant Name	New or Known	PANTHER	Polyphen	Published Dysfunction	Severe HTG (n=110)	Controls (n=472)
Gene: *LPL*						
p.Q-12E>11X	New	Frameshift with early truncation			1 (11.4)*	0
p.D25H	New	Probable	Probable	None	1 (33.4)	0
p.W86R	Known	Probable	Probable	Causative for HLP type 1 (ref. 28); <3% specific activity of WT *LPL in vitro*	1 (44.1)	0
p.G188E	Known	Possible	Possible	Causative for HLP type 1 (ref. 26); <1% specific activity of WT *LPL in vitro*	2 (17.7; 54.7)	0
p.I194T	Known	Possible	Probable	Causative for HLP type 1 (ref. 27); <1% specific activity of WT *LPL in vitro*	1 (35.7)	0
p.P207L	Known	Probable	Probable	Causative for HLP type 1 (ref. 29); <1% specific activity of WT *LPL in vitro*	1 (34.8)	0
Gene: *APOC2*						
p.K19T	Known	Possible	Possible	Associated with dyslipidemia (ref. 30,31)	3 (44.2; 15.1; 22.6)	1 (2.0)
IVS2-30G>A	New	Splicing mutation		None	1 (11.4)	0
Gene: *APOA5*						
p.A315V	Known	Possible	Probable	None	1 (41.8)	0

*Maximum recorded plasma triglyceride concentration (in mmol/L) for the carrier(s) of the specified mutation.

HTG indicates hypertriglyceridemia; *LPL*, gene encoding lipoprotein lipase; *APOC2,* gene encoding apolipoprotein C-II; *APOA5*, gene encoding apolipoprotein A-V; HLP, hyperlipoproteinemia; WT; wild-type gene product.

rious; however, in the absence of more definitive demonstration of dysfunction, we treated the single carrier of this mutation as a noncarrier in subsequent analyses.

Common DNA Sequence Variants

By resequencing, we found 5 reported candidate gene single nucleotide polymorphisms (SNPs) with MAF >1% in controls: 3 in *LPL*, namely p.D9N, p.N291S, and p.S447X and 2 in *APOA5*, namely p.S19W and p.V153 mol/L (Table 3). The *LPL* SNPs were previously functionally assessed: p.D9N had compromised LDL uptake but not impaired hydrolysis,[33] whereas p.N291S[34] and p.S447X[35] had ≈50% decreased and ≈30% increased hydrolytic capacity, respectively. *APOA5 p.S19W* is defectively secreted in vitro.[36] The *APOA5* p.S19W allele was significantly more prevalent in severe HTG cases compared with controls (Table 3).

Differences in Distribution of DNA Variants Between Cases and Controls

Frequencies of carriers of rare mutations in severe HTG patients and normotriglyceridemic controls are shown in Table 4. Heterozygous *LPL* mutations p.Q-12E-11X (once), p.D25H (once), p.W86R (once), p.G188E (twice), p.I194T (once), and p.P207L (once) were present cumulatively in 7/110 (6.4%) of severe HTG patients compared with 0/472 controls; the carrier odds ratio (OR) was infinite ($P<0.00001$). When heterozygotes for either *APOC2* p.K19T or IVS2–30G>A were included, 10.0% of severe HTG patients compared with 0.2% of controls were carriers of mutations (carrier OR 52, 95% confidence interval [CI] 8.6 to 319; $P<10^{-7}$).

The *APOA5* p.S19W loss-of-function allele was strongly associated with severe HTG: 34.6% of HTG subjects were carriers versus 8.8% of controls (OR 5.5, 95% CI 3.3 to 9.1; $P<10^{-9}$). The *LPL* p.D9N variant was modestly associated with severe HTG: 10.9% of cases were carriers versus 3.6% of controls (OR 3.2, 95% CI 1.5 to 7.0; $P=0.0017$). The *LPL* p.S447X variant had a borderline associated with protection from severe HTG: 6.4% of HTG subjects were carriers versus 10.8% of controls (OR 0.44, 95% CI 0.20 to 0.99; $P=0.043$). To quantify the total genetic contribution of the most significantly associated variants, we determined carrier OR for subjects with ≥1 copy of either the heterozygous rare mutations or ≥1 copy of the *APOA5* p.S19W allele. We found that 41.8% of HTG subjects were carriers compared with 8.9% of controls (OR 7.4, 95% CI 4.5 to 12.0; $P<10^{-13}$).

Table 3. Common DNA Sequence Polymorphisms in *LPL*, *APOC2* and *APOA5* in Patients With Severe Hypertriglyceridemia

Variant Name	Novel or Known	Published Evidence for Dysfunction	Allele Frequency	
			Severe HTG (n=110)	Controls (n=472)
Gene: *LPL*				
p.D9N	Known SNP	5-fold enhanced LDL internalization in vitro (ref. 33)	0.054	0.018*
p.N291S	Known SNP	associated with dyslipidemia (ref. 34); ≈60% specific activity of WT LPL in vitro	0.020	0.025
p.S447X	Known SNP	"gain of function" variant (ref. 35); ≈130% specific activity of WT LPL in vitro	0.036	0.068*
Gene: *APOA5*				
p.S19W	Known SNP	associated with dyslipidemia (ref. 36–39); 50% reduced secretion from cultured liver cells	0.204	0.046†
p.V153m	Known SNP	none	0.009	0.011

†$P<0.0001$; *$P<0.05$.
Abbreviations as in TABLE 2; SNP indicates single nucleotide polymorphism.

Response to Fibrate Therapy According to Genotype

To determine a possible between-genotype difference in plasma TG response to oral fibrate treatment, we performed an exploratory post hoc analysis in the subgroup of 53 nondiabetic HTG patients whose treatment consisted only of dietary counseling and usual doses of fenofibrate, gemfibrozil, or bezafibrate as monotherapy. In this subgroup (50.1±12.9 years, 34% female), we recorded the maximal percent change from baseline plasma lipoproteins within 12 months of initiating treatment. The subgroup comprised 7, 18, and 28 HTG patients who had ≥1 copy of either the heterozygous rare mutations, ≥1 copy of the *APOA5* p.S19W allele, and neither, respectively. We observed a significant difference in plasma TG response between genotypes: patients who had ≥1 copy of the rare mutations had a blunted maximal decrease in plasma TG compared with other subjects (Figure). We observed significant between-group differences in increased plasma HDL cholesterol but no difference in total cholesterol on treatment (Figure).

Table 4. Carrier Frequencies for DNA Variants Found in This Study

	Severe HTG	Controls	*P* Value
Rare variants (control MAF<1%)			
≥1 *LPL* mutation	6.4%	0%	$<10^{-5}$
≥1 *APOC2* mutation	3.6%	0.2%	0.0005
≥1 *APOA5* mutation	0.9%	0%	0.038
≥1 *LPL* or *APOC2* mutation*	10.0%	0.2%	$<10^{-7}$
Common variants (control MAF>1%)			
≥1 *LPL* p.D9N allele	10.9%	3.6%	0.0017
≥1 *LPL* p.N291S allele	3.7%	5.5%	NS (0.43)
≥1 *LPL* p.S447X allele	6.3%	13.4%	0.042
≥1 *APOA5* p.S19W allele	34.6%	8.8%	$<10^{-9}$
≥1 *APOA5* p.V153M allele	3.0%	2.1%	NS (0.17)
Rare plus common variants			
≥1 rare mutation or ≥1 *APOA5* p.S19W allele	41.8%	8.9%	$<10^{-13}$

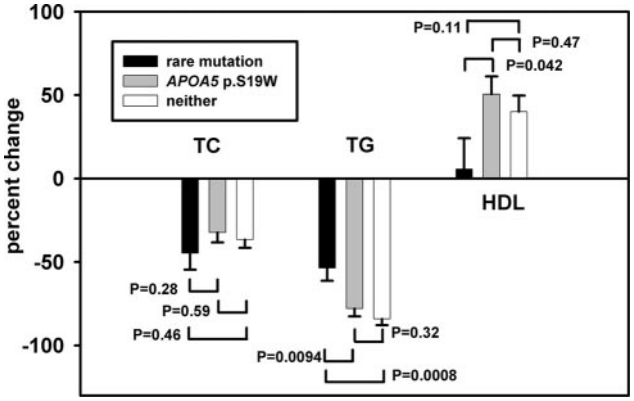
*Individual with *APOA5* p.A315V was treated as a control, given the uncertainty about the dysfunction of this variant (see Results section).
Abbreviations as in TABLE 3; MAF indicates minor allele frequency.

Discussion

By resequencing 3 candidate genes, we found an association of several genetic variants with severe HTG in Canadian patients of European ancestry. Specifically, we found that 41.8% of subjects with plasma TG >10 mmol/L have ≥1 copy of several rare coding sequence variants in candidate genes (*LPL* or *APOC2*) or ≥1 copy of the common *APOA5* p.S19W allele, whereas this assortment of genetic variants is present in only 8.9% of controls (OR 7.4, 95% CI 4.5 to 12.0; $P<10^{-13}$). This is among the most substantial genetic contribution yet detected for a dyslipoproteinemia phenotype. We also observed that carriers of ≥1 copy of several rare mutations in *LPL*, *APOC2*, or *APOA5* genes had a smaller decrease in plasma TG in response to oral fibrate treatment than subjects with other genotypes.

Previous studies of patients with extreme lipoprotein phenotypes showed that rare candidate gene mutations are present in a significant minority of cases. For instance among patients with low HDL, ≈16% had candidate gene mutations compared with ≈2% of controls.[13] Our findings support a similar significant contribution of rare candidate gene mutations in a minority of patients with severe HTG: rare mutations were seen in 10.0% of severe HTG patients compared with 0.2% of controls were carriers of mutations (OR 52, 95% CI 8.6 to 319; $P<10^{-7}$). In addition, we also found a very strong association of severe HTG with the common dysfunctional *APOA5* p.S19W variant: 34.6% of HTG subjects were carriers versus 8.8% of controls (OR 5.5, 95% CI 3.3 to 9.1; $P<10^{-9}$). Thus, the genetic component of this complex metabolic trait is comprised of both rare and common variants, which together account for a greater proportion of affected individuals (>40% in this sample) than rare mutations alone.

The present findings quantify the potential contribution of mutant *LPL* to type 5 hyperlipoproteinemia and solidify a key physiological role for apo A-V, which was only discovered 5



Plasma lipoprotein response (percent change from baseline) in nondiabetic patients with severe hypertriglyceridemia treated with fibrate monotherapy. Patients were stratified by genotype: 7 had ≥1 copy of rare heterozygous mutations (black), 18 had ≥1 copy of the *APOA5* p.S19W allele (gray), and 28 had no genetic variant (white). Mean and standard error of maximal lipoprotein changes from baseline and significance levels for between-genotype differences are shown.

years ago using bioinformatic analysis.[37] The *APOA5* p.S19W allele has been evaluated in several studies,[32] some of which have shown modest associations with mildly elevated plasma TG. Recently, plasma apo A-V concentrations and p.S19W allele frequency were shown to be elevated in patients with relatively mild TG elevation.[38] Among numerous SNPs at the *APOA5* locus, p.S19W is unique because it: (1) alters the amino acid sequence and has proven dysfunction in vitro[36]; (2) is relatively common, with an allele frequency of 7% to 11% in control samples of European ancestry[18,39]; and (3) is the defining polymorphism of a unique haplotype associated with moderately elevated TG.[37,39] Together, the data would indicate that *APOA5* p.S19W might be a clinically useful risk marker of this extreme phenotype.

We selected *LPL*, *APOC2*, or *APOA5* as candidate genes for association with type 5 hyperlipoproteinemia because homozygous mutations in each cause severe HTG with chylomicronemia and especially type 1 hyperlipoproteinemia (MIM 238600), a disorder whose genetic basis is well understood.[3,15] Most *LPL* mutations that we found in severe HTG patients were already proven to be disease-causing in the homozygous state, with documented functional impairment.[26–29] The novel mutations were by and large imputed to have functional impact through their statistical association with severe HTG, their virtual absence from the control sample and also through bioinformatic analysis. Although the findings clearly link heterozygosity for these rare mutations with severe HTG, other factors must be important—both in severe HTG patients with and without the heterozygous mutations.

In the premolecular era, analysis of pedigrees of probands with familial chylomicronemia and biochemical deficiency of LPL suggested that obligate heterozygote carriers of presumed *LPL* mutations had an increased risk of combined hypercholesterolemia and hypertriglyceridemia.[40] Similarly, analysis of pedigrees of probands with familial chylomicronemia attributable to mutant apoC-II isoforms suggested that obligate heterozygote carriers of *APOC2* mutations had elevated plasma TG and high TG content in lipoprotein subfractions.[41] Finally, heterozygous relatives of probands homozygous for truncating mutations in *APOA5* and severe HTG were variably found to have moderately elevated plasma TG.[42,43] Thus, evidence from the pre- and post-genomic eras, including findings from this study, indicates that heterozygosity for rare dysfunctional coding sequence mutations is strongly associated with severe HTG.

Besides *APOA5* p.S19W, among the other coding variants with MAF >1%, *LPL* p.D9N, and p.S447X were found to be associated with susceptibility and protection from HTG, respectively. We noted that the presence of the so-called "gain-of-function" *LPL* p.S447X variant[35] was not "protective" for 7 carriers among the severe HTG patients. Such anecdotal cases may be important considering that p.S447X variant is being proposed as the central component of a gene therapy strategy designed to treat patients with severe HTG.[35]

Thus, our findings are consistent with the emerging model that the cumulative contributions of multiple rare alleles with large genetic effects are found among individuals at the extremes of a complex genetic trait.[13,44] However, in contrast to findings from studies of patients at extremes of very low HDL and LDL cholesterol in which no single variant had a frequency >5%,[12–14] our findings indicate that a variety of low frequency mutations together with the common *APOA5* p.S19W polymorphism, underlie an increased risk in a large segment of patients with extremely high plasma TG. We do not suggest that the heterozygous mutations identified here are directly causative because hyperlipoproteinemia type 5 is a complex trait with no single simple genetic cause and other factors, both genetic and nongenetic are likely to be very important. We also showed a difference in plasma TG response to fibrates, with carriers of ≥1 copy of several rare coding sequence variants in either *LPL*, *APOC2* or *APOA5* having a significantly less favorable response compared with other subjects. The findings further confirm that the genetic contribution to severe HTG is complex and suggest that other genes may still have an important role to play.

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Disclosures

None.

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