

Published in final edited form as:

Nat Genet. 2010 August ; 42(8): 684–687. doi:10.1038/ng.628.

Mutation skew in genes identified by genome-wide association study of hypertriglyceridemia

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Abstract

Genome-wide association studies (GWAS) have replicably identified multiple loci associated with population-based plasma lipid concentrations¹⁻⁵. Common genetic variants at these loci together explain <10% of the total variation of each lipid trait^{4,5}. Rare variants of individually large effect may contribute additionally to the “missing heritability” of lipid traits^{6,7}, however it remains to be shown to what extent rare variants will affect lipid phenotypes. Here, we demonstrate a significant accumulation of rare variants in GWAS-identified genes in patients with an extreme phenotype of abnormal plasma triglyceride (TG) metabolism. A GWAS of hypertriglyceridemia (HTG) patients revealed that common variants in *APOA5*, *GCKR*, *LPL* and *APOB* genes were associated with the HTG phenotype at genome-wide significance. We subsequently resequenced protein coding regions of these genes and found a significant burden of 154 rare missense or nonsense variants in 438 HTG patients, in contrast to 53 variants in 327 controls ($P=6.2 \times 10^{-8}$); this corresponds to a carrier frequency of 28.1% of HTG patients and 15.3% of controls ($P=2.6 \times 10^{-5}$). Many rare variants were predicted *in silico* to have compromised function; additionally some had previously

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Author Contributions. Manuscript and experiment conceptualization: CTJ and RAH. Project management: CTJ and JW. GWAS and statistical analysis: CTJ and MBL. Sequencing: JW, HC, ADM, RAM, RGH and CTJ. Biochemical analysis: MWH. Clinical database management: MRB and BAK. Study samples contributions: RAH, SSA, SY, MEV, GMD SMS, BFV, RE, VS, CJO and SK.

demonstrated dysfunctionality *in vitro*. Rare variants in these 4 genes explained 1.1% of total variation in HTG diagnoses. Our study demonstrates a marked mutation skew that likely contributes to disease pathophysiology in patients with HTG.

Genome-wide association studies (GWAS) have replicably identified novel and known loci associated with population-based plasma lipid concentrations¹⁻⁵. Despite the robustness of these associations, the proportion of variability explained by GWAS-identified loci is relatively modest, <10% in most studies^{4,5}. While vastly expanded study sample sizes continue to reveal new associations, each newly associated variant has an incrementally smaller effect size and contributes only marginally to the cumulative variation of each lipid phenotype⁶. This suggests that GWAS of population-based subjects may be reaching their limits to explain genetic variation of complex traits. A question that has arisen is whether additional forms of genetic variation, such as rare variants of individually large effect, could contribute to the “missing heritability” of complex traits such as plasma lipid concentrations^{6,7}. While the mechanistic basis for the association between lipid traits and most common variants discovered in GWAS is still largely unknown, it remains possible that rare variants in GWAS-identified genes may contribute significantly to lipid phenotypes.

Studying subjects at the extremes of a quantitative phenotype distribution has proven useful to identify functional rare variants⁸⁻¹². Using missense-accumulation analysis in genes defined *a priori* as likely to contain rare variants, a burden of mutations can be quantified statistically in subjects with severe phenotypes, prior to functional assessment of each variant. Primary hypertriglyceridemia (HTG) is one such complex polygenic disease broadly defined by fasting plasma TG concentrations >95th percentile¹³. Resequencing of TG-modulating candidate genes has implicated both common and rare variants in HTG disease pathophysiology^{9,14-16}, however the majority of phenotypic variation underlying severe HTG remains unattributed¹⁷. Our objectives were: 1) to perform a non-biased GWAS of patients with HTG to identify common variants associated with HTG; and 2) to resequence coding regions of candidate genes in loci reaching genome-wide significance to evaluate the burden of rare variants in HTG patients compared with controls. Here, we demonstrate that loci found to be associated with HTG by GWAS using common variants also harbour a significant excess of rare variants.

In total, 555 HTG patients and 1319 controls were included in two cohorts of the study: the GWAS cohort included 463 HTG patients and 1197 controls and the sequencing cohort included 438 HTG patients and 327 controls. HTG patients were unrelated subjects diagnosed with Fredrickson hyperlipoproteinemia (HLP) phenotypes 2B (MIM 144250), 3 (MIM 107741), 4 (MIM 144600) or 5 (MIM 144650), ascertained primarily from a single tertiary referral lipid clinic. The mean plasma TG concentration of HTG patients was 14.3 mmol/L. Controls had maximum recorded fasting plasma TG concentrations <2.3 mmol/L to exclude undiagnosed HTG. All study subjects were of self-declared European ancestry; subjects deviating from European ancestry as determined by multi-dimensional scaling using whole-genome SNP data were removed from sequencing analysis (Supplemental Fig. 1). As expected, clinical characteristics of HTG patients were less favorable than controls, with worse lipid profiles and an increased prevalence of type 2 diabetes (Table 1).

The HTG phenotype was tested for association with >2.1 million single nucleotide polymorphisms (SNPs) using an additive multivariate logistic regression model (Supplemental Fig. 2). This model appropriately adjusted for sex, body mass index, diabetes status and 10 principal components of ancestry (Supplemental Fig. 3). Four loci were significantly associated with HTG at genome-wide significance: *APOA5*, *GCKR*, *LPL* and *APOB* (Table 2). Most associations with HTG were mediated by the same genomic loci

associated with fasting plasma TG concentration in population-based GWAS⁵: *APOA5* and *GCKR* were associated at the same lead SNP, and *LPL* was associated with the same haplotype block. Conversely, the HTG-associated SNPs in *APOB* were ~123-kb upstream of the gene, perhaps consistent with the involvement of regulatory elements in the over-expression of TG-rich lipoproteins in HTG pathophysiology. Investigation of sub-threshold association signals did not provide any additional insight into novel HTG-associated genes.

Subsequently, we tested the hypothesis that common genetic variants in remaining known TG-associated loci are similarly associated with HTG⁵. Only three loci were replicated at a Bonferroni-corrected significance threshold of $P < 0.005$: *MLXIPL*, *TRIB1*, and *ANGPTL3* (Table 2). Positive replication of these TG-associated loci, combined with trends towards significance at *FADS* ($P = 0.05$) and *NCAN* ($P = 0.07$), suggest that additional TG-modulating loci may also be involved in HTG pathophysiology, however smaller effect sizes likely limit their detection.

We next hypothesized that HTG-associated genes would harbour rare variants related to HTG disease causation. The protein-coding sequences of *APOA5*, *GCKR*, *LPL* and exons 26 and 29 (67.8%) of *APOB* were resequenced in individual subjects as regions most likely to harbour protein-compromising mutations. Across the 4 genes, 80 distinct rare variants were identified with minor allele frequencies $< 1\%$ in controls (Fig. 1, Supplemental Table 1). A significant accumulation of rare variants was identified in HTG patients (Table 3), including 154 total variants in 438 HTG diploid genomes compared to 53 total variants in 327 control diploid genomes ($P = 6.2 \times 10^{-8}$), corresponding to a significantly increased carrier frequency of 28.1% in HTG patients versus 15.3% in controls ($P = 2.6 \times 10^{-5}$). A more restricted analysis of rare variants found exclusively in either HTG patients or controls, deliberately removing all reported variants without demonstrated functional compromise, similarly revealed a significant burden of 47 variants in HTG patients compared to 9 variants in controls ($P = 2.4 \times 10^{-5}$); this corresponds to a significantly increased carrier frequency of 10.3% in HTG patients compared to 2.8% in controls ($P = 4.4 \times 10^{-5}$). HTG carriers' fasting plasma TG concentrations ranged from 3.10–88.5 mmol/L, whereas control carriers' fasting plasma TG concentrations ranged from 0.45–1.93 mmol/L. No discernable patterns were observed between such attributes as the gene, mutation type or mutation position with plasma TG concentration or HTG phenotype.

The strength of association between HTG and genomic loci did not predict the mutation accumulation observed in the resequenced genes. *LPL* harboured the largest relative proportion of rare variants, followed by *GCKR*, *APOB* and *APOA5*, with 30.9, 10.7, 9.3, and 4.5 rare variants per kilobase of coding sequence in HTG patients, whereas the same genes harboured 5.6, 2.7, 4.3 and 0.9 rare variants per kilobase of coding sequence in controls. The burden of rare variants found in HTG patients is highly suggestive of phenotype causation, supported by several truncation mutations, *in silico* predictions of deleterious effects, and bonafide characterized deleterious mutations (Supplemental Table 1). The majority of subjects carried only 1 rare variant, however subjects with multiple rare variants were also significantly over-represented in HTG patients (6.6% HTG carriers versus 0.9% control carriers; $P = 3.7 \times 10^{-5}$). Not all rare variants in HTG patients are necessarily sufficient to cause HTG, however they likely contribute to the biochemical heterogeneity observed between patients. For instance, the *APOB* R3500W variant causes hypercholesterolemia¹⁸, but was found in a HTG patient with Fredrickson HLP phenotype 2B, defined by both plasma TG and total cholesterol in excess of the 95th percentile. For this individual case, *APOB* R3500W is more likely contributing to the elevated total cholesterol phenotype, but the mutation is part of the genetic background of this patient that led to his ascertainment through the lipid clinic. This patient exemplifies our working model that both common and

rare genetic determinants in TG-associated genes together contribute to the phenotypic heterogeneity underlying HTG.

Finally, we assessed the contribution of genetic and clinical variables to the total variation in HTG diagnosis, in subjects common between GWAS and sequencing cohorts. A comprehensive logistic regression model including clinical variables and both common and rare genetic variants explained 41.6% of total variation in HTG diagnosis: clinical variables explained 19.7%, common genetic variants in 7 HTG-associated loci explained 20.8% and rare genetic variants in 4 HTG-associated loci explained 1.1%. These data suggest that rare variants found in 4 GWAS-identified genes incrementally contribute to the unexplained variation underlying HTG pathophysiology.

In summary, we performed a GWAS and resequencing of HTG-associated genes and found a significant accumulation of missense and nonsense mutations that contribute to the unexplained genetic component of HTG. Our results suggest that a complex genetic architecture of both common and rare variants in a spectrum of TG-associated genes is responsible for HTG. Future studies using high-throughput next generation sequencing are required to determine whether these associations extend to additional HTG-associated genes, including *MLXIPL*, *TRIB1*, and *ANGPTL3*, and TG-associated genes identified by epidemiological-scale GWAS of population-based samples. It also remains possible that rare variants in TG-modulating genes that have not yielded signals on GWAS, such as *GPIHBP1* or *LMF1*, will further contribute to HTG phenotypes^{19,20}. Functional analyses may more accurately define the extent of dysfunction of rare variants identified in HTG patients and their role in disease causation, while higher level analyses including gene-gene and gene-environment interactions will determine the combined impact of multiple genetic variants on plasma TG concentration in patients with HTG. Our study demonstrates that an accumulation of rare variants is present in GWAS-identified genes and that these contribute to the missing heritability of complex traits among individuals at the extreme of a lipid phenotype.

Methods Summary

Study Subjects

HTG patients were ascertained through tertiary referral lipid clinics. Controls were obtained from population-based studies including the Study of Health Assessment and Risk in Ethnic Groups²¹ and the Myocardial Infarction Genetics Consortium²² (Supplemental Methods). Biochemical analyses were conducted separately in each cohort, as previously described^{14,21,22}.

GWAS

All subjects were genotyped using Affymetrix Genome-Wide Human SNP Array 6.0 (Affymetrix, Santa Clara, CA) according to protocols of the London Regional Genomics Centre (www.lrgc.ca) or the Broad Institute (<http://www.broadinstitute.org/>). Imputation was conducted using HapMap CEU phased haplotypes in MACH²³. All genotypes were filtered for minor allele frequency >1%, Hardy Weinberg $P > 0.0001$, and 95% call rate or imputation quality $r^2 > 0.4$. Identity-by-state calculations, multi-dimensional scaling, and association testing were conducted in PLINK²⁴. Genome-wide significance was pre-specified as $P < 5 \times 10^{-7}$; nominal significance for replication of known TG-associated SNPs was a Bonferroni-corrected threshold $P < 0.005$. Covariates entered into all analyses included sex, body mass index, diabetes status and 10 principal components of ancestry as generated by Eigenstrat^{25,26}.

Sequencing and Mutation Accumulation

All genes were bidirectionally sequenced in individual samples using an ABI 3730 Automated DNA Sequencer and called using automated software (Applied Biosystems, Foster City, CA). Rare variants were manually curated, confirmed by repeat analysis, and annotated *in silico* for functional effects using Polyphen (<http://genetics.bwh.harvard.edu/pph/>). Only rare variants <1% in controls causing missense or nonsense mutations were counted towards mutation accumulation. Rare variant accumulation was compared between HTG patients and controls using Fisher's exact test, defining nominal significance as a 2-sided $P < 0.05$ (Supplemental Methods).

Explained Variation

Total explained variation was calculated from the residuals of multivariate logistic regression model including age, sex, body mass index, diabetes status, and HTG-associated common and rare variants as independent variables, using a SAS v9.2 (SAS Institute, Cary, NJ) macro written for this purpose²⁷ (Supplemental Methods).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the London Regional Genomics Centre (David Carter, Gerry Barbe, and Kevin Kang) for their dedication to this project. We also thank the Myocardial Infarction Genetics Consortium (MIGen) study for the use of their genotype data as control data in our study. The MIGen study was funded by the U.S. National Institutes of Health and National Heart, Lung, and Blood Institute's STAMPEED genomics research program (R01 HL087676) and a grant from the National Center for Research Resources (U54 RR020278). This work was made possible by the facilities of the Shared Hierarchical Academic Research Computing Network (SHARCNET; www.sharcnet.ca). CTJ is supported by a CIHR Banting and Best Canada Graduate Scholarship, a Heart and Stroke Foundation Program Grant, and a CIHR Vascular Research Fellowship. VS was supported by the Sigrid Juselius Foundation and by the Finnish Academy (grant #129494). SSA is supported by the Michael G. DeGroot Heart and Stroke Foundation of Ontario Chair and the Eli Lilly May Cohen Chair in Women's Health Research at McMaster University. RAH is supported by the Jacob J. Wolfe Distinguished Medical Research Chair, the Edith Schulich Vinet Canada Research Chair in Human Genetics (Tier I), the Martha G. Blackburn Chair in Cardiovascular Research, and operating grants from the Canadian Institutes for Health Research (MOP-13430, MOP-79523, CTP-79853), the Heart and Stroke Foundation of Ontario (NA-6059, T-6018, PRG-4854), the Pfizer Jean Davignon Distinguished Cardiovascular and Metabolic Research Award, and Genome Canada through the Ontario Genomics Institute.

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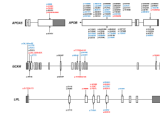


Figure 1. Rare variants identified by resequencing GWAS-identified genes in HTG patients and controls

Variants above gene maps were identified in HTG patients and variants below gene maps were identified in controls. Rare variants are coloured according to their identification in control subjects or previous identification in subjects of unknown clinical status (black), exclusivity to HTG patients or controls (blue), or proven biological dysfunction or truncation (red). Nomenclature refers to functional protein sequences. Only exons 26 and 29 were resequenced in *APOB*. Gene maps are roughly to scale, although differ in scale between genes. GWAS, genome-wide association study; HTG, hypertriglyceridemia; TG, triglyceride.

Table 1

Baseline clinical attributes of the study sample.

	GWAS cohort		Resequencing cohort	
	HTG	Controls	HTG	Controls
Number	463	1197	438	327
Female	30.7%	40.4%	33.2%	50.5%
Diabetes	25.7%	0.4%	28.1%	4.1%
Age (years)	50.9 ± 13.0	47.8 ± 11.1	51.3 ± 13.1	49.9 ± 15.1
Body mass index (kg/m ²)	29.9 ± 4.9	26.4 ± 4.6	30.0 ± 4.9	26.8 ± 4.5
Plasma total cholesterol (mmol/L)	8.2 ± 3.9	5.3 ± 1.3	8.7 ± 4.3	4.9 ± 0.8
Plasma HDL cholesterol (mmol/L)	0.9 ± 0.3	1.4 ± 0.4	0.9 ± 0.3	1.3 ± 0.4
Plasma LDL cholesterol (mmol/L)	-	3.4 ± 1.2	-	3.2 ± 0.9
Plasma triglycerides (mmol/L)	14.3 ± 19.8	1.1 ± 0.7	14.2 ± 19.0	1.2 ± 0.4

GWAS, genome-wide association study; HDL, high-density lipoprotein; HTG, hypertriglyceridemia; LDL, low-density lipoprotein. There are 346 HTG patients and 205 low triglyceride controls common between both cohorts. Lipid measurements were conducted after a 12-h fasting period. Values are mean ± standard deviation. LDL cholesterol is not accurately calculated using the Friedewald equation for HTG patients when plasma triglyceride concentration exceeds 4.5 mmol/L.

Table 2

Genetic loci associated with HTG.

Locus	SNP	CHR	Position	Minor Allele	HTG MAF	Control MAF	OR (95% CI)	P-value
<i>APOA5</i>	rs964184	11	116.2	G	0.33	0.14	3.28 (2.61-4.14)	5.4×10^{-24}
<i>GCKR</i>	rs1260326	2	2.8	T	0.52	0.41	1.75 (1.45-2.12)	6.5×10^{-9}
<i>LPL</i>	rs7016880	8	19.9	C	0.03	0.10	0.32 (0.21-0.49)	2.0×10^{-7}
<i>APOB</i>	rs4635554	2	21.2	G	0.39	0.31	1.67 (1.38-2.02)	2.0×10^{-7}
<i>MLXIPL</i>	rs714052	7	72.5	G	0.07	0.13	0.44 (0.31-0.62)	0.000003
<i>TRIB1</i>	rs2954029	8	126.6	T	0.37	0.46	0.71 (0.59-0.86)	0.0004
<i>ANGPTL3</i>	rs10889353	1	62.9	C	0.27	0.32	0.73 (0.59-0.89)	0.002
<i>NCAN</i>	rs17216525	19	19.5	T	0.07	0.09	0.71 (0.50-1.00)	0.05
<i>FADS</i>	rs174547	11	61.3	C	0.40	0.33	1.20 (0.99-1.44)	0.07
<i>XKR6</i>	rs7819412	8	11.1	G	0.46	0.50	0.87 (0.72-1.05)	0.14
<i>PLTP</i>	rs7679	20	44.0	C	0.20	0.19	1.17 (0.94-1.47)	0.16

CHR, chromosome; CI, confidence interval; HTG, hypertriglyceridemia; MAF, minor allele frequency; OR, odds ratio; SNP, single nucleotide polymorphism. Association was tested using an additive multivariate logistic regression model, entering sex, body mass index, diabetes status, and 10 principal components of ancestry as covariates. The top four loci surpassed a pre-specified threshold for genome-wide significance of $P < 5 \times 10^{-7}$. Remaining loci were replicated from GWAS meta-analysis of population-based TG concentrations⁵, using a Bonferroni corrected significance threshold of $P < 0.005$.

Table 3

Rare variant accumulation identified by resequencing GWAS-identified genes in HTG patients and controls.

	All mutations		Missense/Indels		Nonsense	
	HTG	Controls	HTG	Controls	HTG	Controls
Total alleles	876	654	876	654	876	654
All Variants						
<i>APOA5</i>	5	1	3	1	2	0
<i>GCKR</i>	20	5	14	4	6	1
<i>LPL</i>	44	8	43	8	1	0
<i>APOB</i>	<u>85</u>	<u>39</u>	<u>84</u>	<u>39</u>	<u>1</u>	<u>0</u>
Total	154	53	146	52	9	1
	P = 6.2 × 10⁻⁸		P = 3.2 × 10⁻⁷		P = 0.051	
<hr/>						
Exclusive Variants						
<i>APOA5</i>	4	0	2	0	2	0
<i>GCKR</i>	9	0	7	0	2	0
<i>LPL</i>	19	2	18	2	1	0
<i>APOB</i>	<u>15</u>	<u>7</u>	<u>14</u>	<u>7</u>	<u>1</u>	<u>0</u>
Total	47	9	42	9	5	0
	P = 2.4 × 10⁻⁵		P = 1.4 × 10⁻⁴		P = 0.075	

GWAS, genome-wide association study; HTG, hypertriglyceridemia. Exclusive variants refer to rare variants found exclusively in HTG cases or low triglyceride controls; previously reported variants without characterized functional compromise are deliberately excluded. Fisher's exact test was used to calculate the significance of rare variant accumulation in HTG patients, defining nominal statistical significance as a two-sided $P < 0.05$. Mutation counts and annotations are found in Supplemental Table 1.